STUDIES ON PLASMA PROTEINS IN LIVER DISEASE USING QUANTITATIVE IMMUNOELECTROPHORESIS

by

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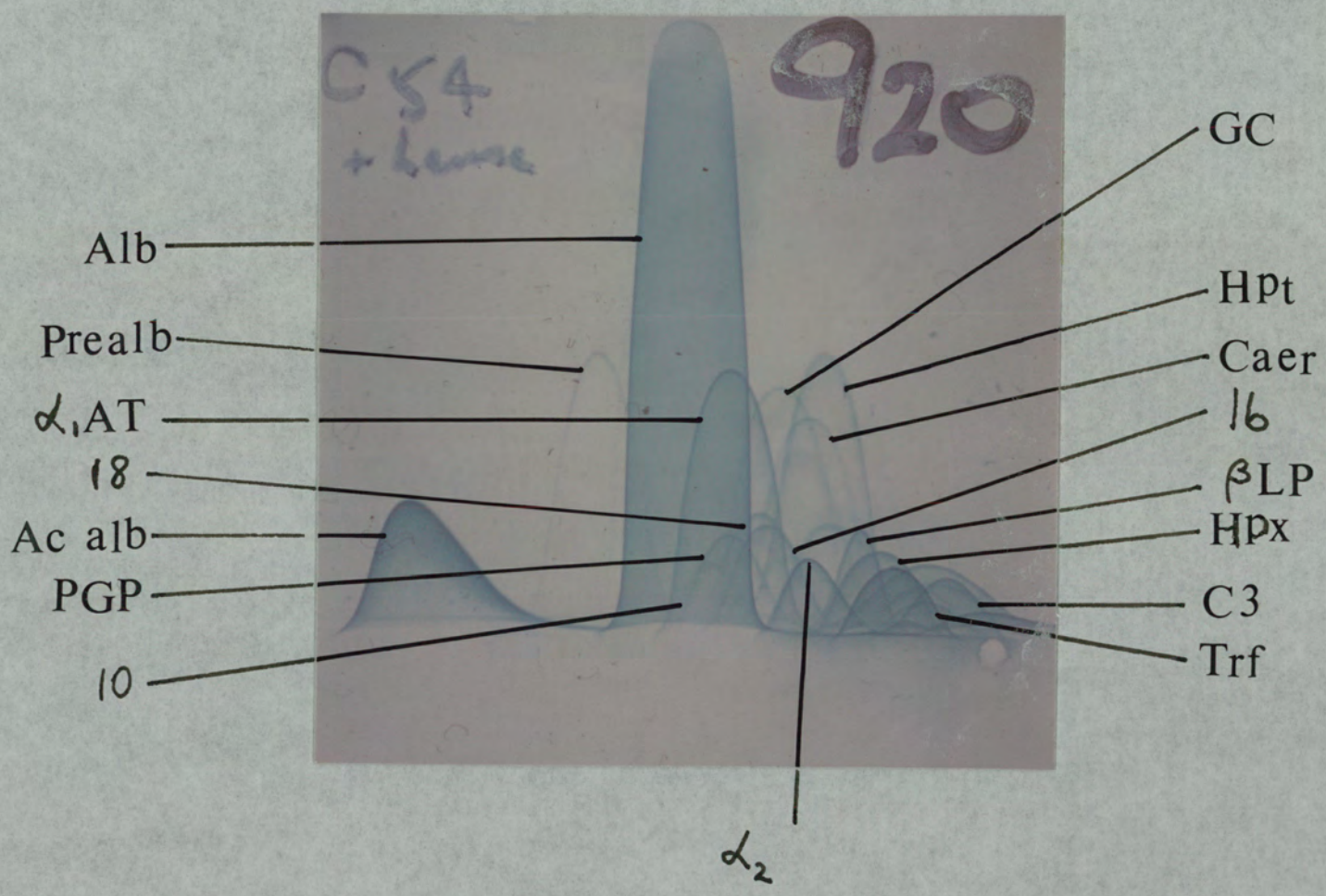
For the King of Babylon stood at the parting of the way, at the head of the two ways, to use divination: he made his arrows bright, he consulted with images, he looked in the liver

Ezekial 21 v. 21



Frontispiece. Crossed immunoelectrophoresis of normal serum on 5 x 5cm. miniplate showing acetylated albumin marker (Ac Alb), prealbumin (prealb), albumin (alb) α_1-antitrypsin (α_1-AT) α_1 easily precipitable glycoprotein (PGP) group component (G.C.) caeruloplasmin (Caer) haptoglobin (Hpt) transferrin (Trf) haemopexin (Hpx) β lipoprotein (βLP) α_2 macroglobulin (α_2M) third component of complement (C3 and proteins number 10, 16 and 18.

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C O N T E N T S

SUMMARY

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1. The history of knowledge on plasma proteins and the methods of separation are reviewed with particular reference to the development of the quantitative immunoelectrophoresis technique used in the present work.

The discovery of plasma protein abnormalities in liver disease and the evidence that the liver is the main site of synthesis is discussed. The mechanisms of plasma protein synthesis and catabolism and their control are outlined.

2. A summary is given of the properties and functions of the 14 plasma proteins studied in this work (prealbumin, α_1-easily precipitable glycoprotein, α_1-antitrypsin, group component, α_2-macroglobulin, caeruloplasmin, haptoglobin, haemopexin, transferrin, β-lipoprotein, β_1A-C globulin (C3, third component of complement) and protein numbers 10, 16 and 18).

3. A quantitative modification (Clarke and Freeman) of Laurell's crossed immunoelectrophoresis method using large plates is described.

4. Clarke and Freeman's method has been adapted for 5 x 5cm. plates (miniplates) with great saving in cost. This miniplate method was shown to give a reproducible and quantitative measure of plasma protein concentration.

5. Aliquots of sera were stored at -20°C. The concentration of β-lipoprotein fell by 30-50% but the levels of other proteins were unchanged over a period of 12 months.

6. Using the miniplate method a normal range for 13 proteins was established in a control population of 70 healthy blood donors and laboratory personnel. Prealbumin was found to be significantly higher in males than females and $\alpha_2$macroglobulin and caeruloplasmin were higher in females. Haemopexin and easily precipitable glycoprotein increased

significantly with age. These findings are in agreement with other published studies. Repeated samples from normal individuals over a period of months showed there was little change in the concentration of any protein.

As few pure protein standards are available, protein concentrations in this study have been expressed as a percentage of a standard serum. However, absolute values for 6 proteins were obtained by reference to a standard serum with known concentrations of these proteins. The normal ranges for these proteins in the control population agree well with other published results.

7. Sera from seven diagnostic groups of patients were examined. Large plates were used to study sera from patients with primary haemochromatosis (29), alcoholic cirrhosis (12), cryptogenic cirrhosis (12) and active chronic hepatitis (20). Miniplates were used for sera from patients with acute viral hepatitis (12), extrahepatic biliary obstruction (12), primary biliary cirrhosis (20) and a further series of patients with haemochromatosis (28).

Statistically significant changes in the concentration of at least one plasma protein were found in all diagnostic groups studied and the most striking and numerous changes occurred in the disease groups with the greatest abnormality of liver function tests.

Prealbumin. Mean levels were low in each of the 4 groups of patients studied (viral hepatitis, extrahepatic obstructive jaundice, primary biliary cirrhosis and haemochromatosis).

Easily precipitable glycoprotein. Mean levels were raised in acute viral hepatitis, extrahepatic obstructive jaundice, primary biliary cirrhosis and active chronic hepatitis but normal in the other diagnostic groups.

Alpha-1-antitrypsin levels. Alpha-1-antitrypsin levels were raised in acute viral hepatitis, extrahepatic obstructive jaundice, and primary biliary cirrhosis but normal in primary haemochromatosis.

Group component. Mean concentrations were normal in all the seven diagnostic groups of patients with liver disease.

Alpha-2-macroglobulin. The mean concentration was raised only in those groups of patients with extensive hepatic fibrosis or cirrhosis.

Caeruloplasmin. The concentration was normal in haemochromatosis and cryptogenic cirrhosis but raised in the other 5 groups.

Protein 10. The concentration was raised in acute viral hepatitis but normal in all other diagnostic groups.

Haptoglobin. The concentration was low in acute viral hepatitis and active chronic hepatitis and no visible precipitation arc was seen in some cases. Levels were normal in the other groups.

Haemopexin. The concentration was low in acute viral hepatitis, extrahepatic obstructive jaundice, and primary biliary cirrhosis but normal in the other diagnostic groups.

Transferrin. The concentration was low in extrahepatic obstructive jaundice, haemochromatosis and alcoholic cirrhosis but raised in active chronic hepatitis.

Beta lipoprotein. The concentration was raised in acute viral hepatitis and reduced in extrahepatic obstructive jaundice but normal in the other groups.

Protein 16. The concentration was raised in extrahepatic obstructive jaundice and primary biliary cirrhosis but normal in acute viral hepatitis and haemochromatosis.

Protein 18. The mean concentration was reduced in extrahepatic obstructive jaundice and the protein was undetectable in several cases. Levels were normal in acute viral hepatitis, primary biliary cirrhosis and haemochromatosis.

Beta 1-AC globulin (C3), was measured only in the large plate studies and was raised in cryptogenic and alcoholic cirrhosis and active chronic hepatitis.

The pattern of changes seen was very similar to the non-specific "acute phase reaction". When there was severe parenchymal liver disease the pattern appeared to be modified by the reduced hepatic capacity for protein synthesis. This was clearly seen in cases of active chronic hepatitis where only after liver function had improved with treatment was the synthetic capacity of the liver increased sufficiently to express the acute phase reaction fully. The major differences from the acute phase reaction as normally seen were normal or low concentrations of haptoglobin thought to be due at least in part to shortened red-cell life span and raised levels of alpha-2-macroglobulin which were found only in liver disease in which there was extensive fibrosis or established cirrhosis.

8. The previously reported finding of raised concentrations of haemopexin and other proteins in primary haemochromatosis was not confirmed in further studies using the miniplate technique, and detailed examination of the discrepancy suggested that a technical error accounted for the results of the earlier study. Serial venesection in 6 patients with haemochromatosis did not significantly effect the concentration of any protein with the exception of transferrin which rose progressively in 3 cases.

Haemopexin concentrations were also normal in close relatives of patients with haemochromatosis and in patients with secondary iron overload. The serum transferrin concentration was significantly reduced in the relatives of patients with haemochromatosis.

9. There was no evidence for in vivo conversion of the third component of complement (C3) in a small number of patients with active chronic hepatitis and primary biliary cirrhosis.

10. The changes in plasma protein concentration following orthotopic transplantation of the liver were followed in 4 patients. The same pattern of changes was seen post-operatively with rejection of the graft, biliary

obstruction and sarcinomatosis and the pattern was similar to the "acute phase reaction". In one case a precipitous fall in C3 occurred at the onset of an acute rejection episode suggesting consumption of complement.

11. In the sera from 7 patients with severe deficiency of alpha-1-antitrypsin deficiency the concentration of the other proteins was normal apart from a rise in α_2-macroglobulin and protein 10.

12. No change occurred in the concentration of the 13 proteins studied over a 7-day period in two normal subjects during hepatic microsomal enzyme induction.

In 15 epileptic patients receiving long-term anti-convulsant therapy, in whom raised urinary glucaric acid excretion indicated hepatic enzyme induction, there were significant increases in the concentration of easily precipitable glycoprotein, α_1-antitrypsin, group component, α_2-macroglobulin, caeruloplasmin and protein 16.

13. The acute phase reaction and the factors controlling its expression are reviewed. The changes in the concentration of the individual proteins in the different categories of liver disease are discussed in relation to the factors known to control their synthesis and catabolism.

14. The diagnostic value of the plasma proteins measured by immunoelectrophoresis was assessed using a linear stepwise discriminant function and a sequential Bayesian function to reclassify the cases in seven diagnostic groups. The overall error using both methods lay between 12 and 34%. No protein was of particular diagnostic value and the error in reclassification was least when the values of all proteins measured were used. The error in reclassification of 85 cases in seven diagnostic groups using data derived only from the cellulose acetate electrophoresis strip was 51%. Diagnostic accuracy was greatest when all the data from immunoelectrophoresis and cellulose acetate electrophoresis was combined.

PART 1

INTRODUCTION

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PART 1

1. History of plasma proteins and their identification

The history of plasma proteins starts with the discovery of nitrogen by Daniel Rutherford who obtained his Doctorate in Medicine in 1772 from the University of Edinburgh for his "Dissertatio inauguralis de Aere Fixo Dicto, aut Mephitico". Magendie (1816) was the first to recognise the importance of nitrogen in food stuffs and from dietary experiments in dogs he concluded that nitrogen containing nutrients were an essential part of the diet. The Dutch chemist Gerard Mulder (1839) examined a series of nitrogen-rich organic compounds including fibrin and egg albumen and concluded that they all contained the same basic elements in the same proportions and he proposed the name protein from the greek verb meaning "I take the first rank" (Liebig 1842). The view that all proteins were chemically identical was generally accepted for some years (Liebig 1842) but Liebig later (1847) argued against this unitary hypothesis on the basis of further analyses.

The earliest attempt to fractionate the serum proteins was by Parnum (1851, 1852) who found that dilution of serum after weak acidification produced a precipitate. He claimed this separated 'Casein' from serum while albumin, a different protein, remained in solution. This technique of separation was later known as iso-electric fractionation. Denis (1856) also described the use of salt precipitation and was the first to use the word globulin in relation to serum proteins. This fraction appears to have consisted of erythrocyte stroma contaminated by precipitated globulin. He thought it contained dissolved 'globules' of blood and for this reason called it globulin. The term albumin ("albumine") had first been used by Fourcroy in 1789 (see Denis 1856) to describe egg white.

Numerous other fractions were described by other authors and the system was simplified by Hoppe-Seyler (1870) who called globulin that fraction which precipitated on dilution following weak acidification and albumin the protein which remained in solution.

There was little further advance in protein chemistry until the end of the 19th Century when there were two further major discoveries. The electro-chemistry of proteins was introduced in 1892 when Picton and Linden observed the displacement of the line of demarcation between a haemoglobin solution and an aqueous solvent under the influence of an electric current. The second major advance was in analytical chemistry and Fischer was able to conclude after several years research (1906) that acid hydrolysis of all proteins studied yielded amino acids, and he deduced that proteins are made up of a large number of α amino acid residues condensed by the formation of an amide link between the α amino group of one molecule and the carboxyl group of another.

The next real advance was due to the work of the Swedes Svedberg and Tiselius in the 1930's. Svedberg (1928) developed the ultracentrifuge and showed that the weight of protein molecules could be deduced from the equilibrium established in a suitable gravitational field between sedimentation and diffusion. Tiselius (1937) developed an apparatus which allowed the study of the movement of protein ions in an electric field. All serum could be separated into 5 components designated γ, γ, β, α and A (albumin) in increasing order of speed of migration towards the anode. In the classical paper ultra-violet absorption was used for detection of the concentration gradients and this was later superseded by measurement of the gradient of the index of the re-fraction. Increasing resolution was also achieved by increasing the time of electrophoresis and altering the pH of the buffers. Both techniques showed the heterogenous nature of serum proteins obtained by fractional precipitation using ammonium sulphate

and iso-electric fractionation but these techniques remain useful as preliminary steps in the separation of proteins.

Cohn (1953) developed a new and highly selective method of protein precipitation by varying the alcohol concentration, salt concentration, and pH and by these measures not only was the separation better but more nearly quantitative.

Tiselius' method of electrophoresis was technically difficult and time consuming and turbid lipaemic serum could not be analysed due to interference with the optical system of the appartus. Improvements in electrophoretic separation followed changes in the supporting medium. Paper electrophoresis was introduced in 1950 (Durrum 1950) and although more convenient the protein bands were indistinct. Electrophoresis in gels has the advantage that adsorption of the protein is less and with starch (Smithies 1955) and polyacrylamide there is also a molecular sieving effect. For most routine purposes cellulose acetate is now used (Kohn 1957). However with these techniques identification and quantitation of individual proteins is difficult or impossible.

More recently techniques have been developed suitable for the isolation of individual proteins. These include ion exchange chromatography (Freeman 1970), exclusion chromatography (Hjerten, 1971) and electrofocussing (Wrigley 1971) but none are applicable to the examination of several different proteins in large numbers of individual sera.

2. Immunochemistry and immunoelectrophoresis

The first application of immunological techniques to the identification and measurement of serum proteins was in 1946 by Oudin (Oudin 1953) who used vertical diffusion of antigen and antibody in a test tube and showed that human serum contained at least 10 components. In 1948 Ouchterlony adapted the method and carried out precipitation reactions

horizontally on a plate of agar. Because of the proximity of the different precipitin lines these methods are not suitable for examining the large numbers of antigens in serum simultaneously and one solution to the problem was to first separate the proteins by electrophoresis. This was first described in 1953 by Grabar and Williams. The serum was separated into its components by electrophoresis in agar and after its completion antibody was placed in a trough which bordered the length of the agar plate. The antigen and antibody diffused towards each other and precipitated in zones of equivalence. Between the arcs of albumin and gamma globulin there were at least 18 distinct proteins. The density of the precipitation lines was higher in certain regions corresponding to the α_1, α_2 and β globulin bands originally described by Tiselius. A micro-modification of the method was introduced by Scheidegger (1955).

Immunoelectrophoresis represented a considerable advance as it clearly showed that the 5 conventional electrophoretic fractions were composed of a large number of individual proteins many of which were identified for the first time. However, accurate quantitation of the proteins was not possible and resolution of the arcs was poor because of the diffusion of the proteins in the second step. Quantitation of individual proteins awaited better methods of separation so that monospecific antisera could be produced. Radial immunodiffusion of the antigen in antibody impregnated agar was introduced by Mancini (1965) and Laurell (1967) used electrophoresis to drive the antigen into the bed of antiserum. This produced a flame shaped track (rocket) and the height of the rocket was proportional to the concentration of the protein in the sample. Comparison of these two methods showed close agreement (Becker 1967). These methods are time consuming and expensive and if immunoelectrophoresis could be made quantitative this should offer a better method of measuring multiple proteins in a single sample.

The first attempt to increase the resolution of protein separation by immunoelectrophoresis was by Ressler (1960) who introduced the concept of two dimensional immunoelectrophoresis. He separated the serum in starch gel and then transferred a strip into a buffer-agar-antiserum mixture for a second dimension electrophoretic run at right angles. Laurell (1965) used the same technique except that agarose was used as the supporting medium for both electrophoretic steps; Agar is a mixture of polysaccarides obtained from algae. Its carbohydrate sequence consists mainly of repeating units of (1-4)-linked 3,6-anhydro-α-L galactopyranose and (1-3)-linked β-D-galactopyranose. It can be separated into two fractions, one of whihh is almost neutral (agarose) and one which is charged due to the content of sulphate groups and carboxyl groups (agaropectin). This implies that because of the fixed nature of the charged agar network there will be water transport towards the cathode (electro-osmosis) with poor separation of the slower moving proteins,and also the risk of interaction with the agar gel (Wieme 1965). These problems are considerably reduced by using agarose.

Laurell's method was modified by Clarke and Freeman (1966; 1968) so as to make it quantitative for the plasma proteins. The whole of the first dimensional strip was used in running the second dimension and they showed that the area under any protein peak was directly proportional to the concentration of that protein in the serum and inversely proportional to the concentration of antibody to that protein in the antiserum to whole human serum. As the titre of antibodies to the different serum proteins differs greatly, the relative sizes of the precipitation arcs for the different proteins are not an indication of their absolute concentration in the serum and different serum samples are compared by reference to a standard serum. This technique and a scaled down modification are used in the present study.

3. Plasma protein disturbances in liver disease

The realisation that disturbances in the plasma proteins occur in liver disease is 70 years old (Jolles 1902) and the finding of a low serum albumin level was soon confirmed by other workers (Gilbert and Chiray 1907; Grenet, 1907). Initially the low albumin was attributed to the sump effect of the albumin-rich ascites but not all cases of cirrhosis with low serum albumin had ascites and Grenet (1907) was later proved correct when he said "nous considérons l'insufficsance hépatique comme capable de déterminer à elle seule une diminution des albumines du sérum sanguin". Later reports also noted a rise in the globulin fraction (Salvesen, 1929; Abrami and Wallich 1929; Peters and Eisenman 1933; Myers and Keefer 1935; Tumen and Bockus 1937).

Detailed observation was delayed until more sophisticated methods of examination of plasma proteins became available. Longsworth et al (1939) were the first to report their results with the Tiselius electrophoresis technique and a flood of papers followed (Luetscher, 1940; Gray and Barron 1943; Sterling et al 1949; Ricketts et al 1949). All noted a fall in albumin and a rise in gamma globulin and in addition in obstructive jaundice there is a rise in beta globulin (Sterling and Ricketts 1949; Kunkel and Ahrens 1949). Results using paper electrophoresis were similar but emphasized the frequent rise in α_1 and α_2 globulins but the patterns were not sufficiently distinctive to differentiate the different types of liver disease with any certainty (Wall, 1958; Osserman and Takatsuki 1963). Initial immunoelectrophoretic studies were also of very limited diagnostic use because the method was not quantitative, but a fall in the concentration of prealbumin and a rise in caeruloplasmin, haptoglobin and α_2 macroglobulin were noted in some patients (Scheidegger and Zahnd, 1957; Bargob et al 1958; Heremans 1959; Izarn et al 1960; Verain et al 1961).

The measurement of certain individual proteins was found to be of some diagnostic value. For instance very low levels of caeruloplasmin were found to be very suggestive of Wilson's disease (Scheinberg and Gitlin, 1952) although low levels may also be found in other types of liver disease. High levels of haptoglobin were in favour of obstructive jaundice rather than parenchymal disease (Owen et al 1961) and genetically determined low levels of α_1 antitrypsin were first recorded in a patient with liver disease in 1967 (Ganrot et al, 1967).

The development of radial immunodiffusion allowed the measurement of several individual plasma proteins in large numbers of patients with liver disease (Muller and Muller von Voigt 1967; Cleve and Strohmeyer 1967) but no attempt was made to use this information in differential diagnosis. Quantitative immunoelectrophoresis is a cheaper and more convenient method of measuring a number of immunologically distinct proteins in the same serum sample and it was chosen for the present studies for this reason.

4. Liver as the Site of Plasma Protein Synthesis

Although the association of liver disease with plasma protein abnormalities had been known for many years, the site of plasma protein synthesis remained unknown until comparatively recently. Kerr et al (1918) noted a definite lag in the restoration of the concentration of serum proteins in Eck fistula dogs following acute plasma depletion or chemical liver injury. Knutti et al (1937) reported observations over 2 years on an Eck fistula dog which at times was unable to maintain normal plasma protein concentration on various standard diets and they presented evidence that its protein synthetic capacity was less than 1/10th normal. At autopsy there were no abnormalities apart from the shrunken liver usually found in Eck fistula animals. In a review of the literature in 1940

Madden and Whipple said "in debate about the source of albumin and globulin hardly an organ or tissue escapes, unless it be the brain".

Tarver and Reinhardt (1947) studied the incorporation of methionine labelled with radio-active sulphur into plasma proteins in the hepatectomised dog and concluded that the majority of albumin, globulin and fibrinogen synthesis occurred in the liver. The identification of the plasma protein fractions containing radio-active label was limited by the techniques available at that time. Miller et al in a series of papers (1951, 1954a, 1954b) provided the first unequivocal evidence that the liver is the major source of newly synthesised albumin, fibrinogen, alpha and beta globulins. Experiments were performed on the incorporation of ^{14}C-lysine into protein by the isolated perfused rat liver; in the initial experiments the fractions were isolated by salt fractionation but in the later work by zone electrophoresis on starch followed by elution of the protein fractions. Perfusion of the anhepatic animal carcass showed that almost all the protein synthesised was gamma globulin. Similar results and conclusions were reached by Kukral et al (1961; 1963) studying the incorporation of ^{35}S-methionine into plasma proteins.

Recently more sophisticated radio-active tracer methods and protein separation systems have demonstrated that the liver is the major if not the only site of the synthesis of most plasma proteins. This evidence will be presented later when the individual plasma proteins are discussed in detail. The principles of the mechanism of plasma protein synthesis will now be illustrated by reference to albumin which has been the most closely studied protein.

5. Mechanism of Plasma protein synthesis

The mechanisms geared for the synthesis of plasma proteins consist of 3 major classes of RNA, messenger RNA (mRNA) ribosomal RNA (r RNA) and

transfer RNA (t RNA) as well as structural protein and the endoplasmic reticulum (ER).

The steps in the synthesis of plasma proteins have been studied most closely for albumin (Munro 1970). The synthesis of albumin takes place in the cytoplasm through the interaction of the ribosome with mRNA and the ER. The ribosome is made up of two subunits. The smaller unit consists of 18S RNA plus protein making a 40S ribosome subunit and the larger consists of 28S RNA plus protein making a 60S subunit. The ribosomal proteins are synthesized in the cytoplasm, then enter into the nucleolus and combine with r RNA to form the specific ribosomal subunits. These then return to the cytoplasm subunit pool and are available for attachment to mRNA. The larger subunit is attached in vertical alignment to the microtubular structure of the ER and it is thought that the growing protein chain enters the ER through the interior space of the 60S subunit.

As each specific amino acyl tRNA, determined by a code of 3 nucleotides in the mRNA, inserts its amino acid into the peptide chain, the peptide is translocated from one spot to the next on the mRNA. The ribosomes move along the mRNA and at the end of the sequence when the albumin polypeptide is completed, the ribosomes are disaggregated from the mRNA and dissociate into subunits mixing with the available old and newly synthesized subunit pool.

Transport through the cell of newly produced albumin molecules has recently been clarified. Peters et al (1971) have shown that following the administration of a tracer dose of labelled amino acid, the specific tRNA becomes labelled through the attachment of the labelled amino acid and the amino acid is incorporated into the growing albumin chain within a minute or less. At 1-3 minutes the labelled albumin reaches the rough endoplasmic reticulum membrane which contains albumin of highest specific activity. At 3-6 minutes the smooth endoplasmic reticulum reaches maximum

specific activity and after 15-20 minutes the Golgi apparatus contains the highest specific activity of albumin. Release of albumin is sensitive to potassium concentration within the liver cell. When potassium content is reduced, or when ouabain is present, the release of labelled albumin is slowed, suggesting an active transport system (Judah and Nicholls, 1970). Geller et al (1972) have presented evidence for the existence of a molecule larger than albumin in the liver cell and suggest that the conversion of this polypeptide to albumin before secretion is rate limiting. The carbohydrate units of the glycoproteins are assembled stepwise by transfer of monosaccharides to the completed polypeptide as it passes along the rough and smooth endoplasmic reticulum (Redman and Cheriam, 1972). The Golgi apparatus probably completes the addition of carbohydrate (Wagner and Cynkin, 1971).

As the plasma proteins are being continuously replaced it is obvious that the plasma concentration represents the balance between synthesis and catabolism and changes in either or both can alter the plasma pool and concentration. It is therefore unwarranted and often wrong to equate a low concentration of a plasma protein with diminished synthesis. This has been clearly shown recently for albumin (Rothschild et al, 1969). In 19 patients hospitalised with cirrhosis and ascites, albumin synthesis measured with the ^{14}C-carbonate technique was found to be normal or elevated in 12 and reduced in only 7. There was no relationship between albumin synthesis and the serum albumin level.

Control of plasma protein synthesis

Quantitative control of the synthesis of any plasma protein can be exercised by recruitment of new cells or by increasing the amount of protein produced by individual cells. Evidence for an increase in the number of parenchymal cells synthesizing a particular protein is given by Lane (1968) and Peters and Alper (1966) for transferrin and haptoglobin respectively using a fluorescent antibody technique. The assumption that the presence

of the protein in the cell is indicative of current synthesis is supported by the observation that binucleate cells stained more frequently than other cells when haptoglobin synthesis was stimulated by turpentine injection. This suggests that some cells may be selectively stimulated to synthesize haptoglobin.

Synthesis in the individual cell can be regulated in many ways. Transcriptional control is effected by altering the rate of formation of mRNA by RNA polymerase from the DNA template or by altering the transport or degradation of mRNA. Translational control acts at a ribosomal level involving the activation of amino acids, their binding onto tRNA and the microsomes and the subsequent events leading to release of the completed protein. The addition of carbohydrate chains to glycoproteins, the cleavage of a precursor molecule before release from the cells and the possible existence of an active transport system for release all offer further post-translational sites at which protein synthesis could be regulated.

Evidence for transcriptional control usually relies on the effect of actinomycin D to suppress the activity of an inducer. Since actinomycin D inhibits DNA-dependent RNA synthesis, inhibition of induction implies that the increased rate of synthesis is dependent on increased levels of mRNA. This appears to be one mechanism by which hepatic enzyme induction by drugs acts (Parke 1971). Evidence will also be given that the augmented synthesis of the acute phase proteins (Neuhaus et al, 1966; Maung et al 1968; Sarcione, 1970) and the induction of caeruloplasmin by copper (Evans et al 1970b) may be mediated in this way. These examples will be discussed later,

There is now good evidence that the state of aggregation of the polyribosomes as assessed on sucrose density gradient analysis is a sensitive index of protein synthesis within the cell (Sidransky 1972). The polyribosomes are readily disaggregated by lack of amino acids, alcohol and other toxins. The effects of fasting on protein synthesis have been

extensively studied and coincident with the disaggregation of the polyribosomes, albumin synthesis rapidly decreases (Miller and John, 1970; Rothschild et al 1972). Refeeding results in reaggregation of the polyribosomes and the synthesis of new albumin, even if actinomycin D is given, suggests that the mRNA for albumin is stable. In the isolated perfused liver taken from a fasted animal the addition of amino acids to the perfusate results in an increase in albumin synthesis to above the levels found in the fed control. This may be because in the fasted state the shorter lived mRNA for other proteins decreases and the long lived mRNA for albumin has less competition for the limited ribosomal population. While overall reduction in amino acids has the maximum effect on protein synthesis, a special role has been suggested for tryptophan, which in addition to its ability to reverse the polyribosome disaggregation in fasting, is also able to reverse the reduction in protein synthesis due to alcohol (Rothschild et al 1971). The exquisite sensitivity to tryptophan may be due to the low levels of the free amino acid in the liver and plasma where most of it is bound to albumin.

Hormones have a complex and poorly understood effect on protein synthesis (Munro 1970). In experimental animals thyroid hormone and cortisone stimulate albumin synthesis in vivo and this effect of corticosteroids has been well documented in patients with liver disease (Cain et al 1970; Westergaard et al 1972). The widespread changes which occur in the concentrations of many plasma proteins in pregnancy and in women on oestrogen containing oral contraceptives is well known. The pattern of changes in these two situations is very similar and quite different from the acute phase reaction (see below). There is a rise in the concentration of α_2-macroglobulin, caeruloplasmin, α_1-antitrypsin and transferrin and a fall in orosomucoid and haptoglobin (Ganrot and Bjerre, 1967; Laurell et al 1967; Horne et al 1970; Mendenhall, 1970; Song et al

1970). Progestagens appear to induce no change in protein concentrations (Laurell et al 1969) but administration of 17-alpha alkyl anabolic steroids is followed by a rise in orosomucoid, α_1-antitrypsin, haptoglobin and prealbumin but no change in transferrin or caeruloplasmin (Barbosa et al 1971). This pattern is different from that produced by both oestrogens and the acute phase reaction.

Protein Catabolism

The factors governing the removal of proteins from the circulation are obviously important determinants of the plasma protein concentration. A 'suicidal' protein like haptoglobin is rapidly cleared from the circulation after combining with haemoglobin and the two molecules of the complex are catabolised together. However it seems that perhaps only half of its catabolism is related to haptoglobin carriage (Noyes and Garby, 1967) and the factors which determine the removal of the other molecules are unknown. Studies with radioactive labelled plasma proteins indicate that for most proteins the catabolic process is indiscriminate and it matters not whether the protein molecules had been present in the circulation for a considerable period of time or had been newly synthesized. Recent work indicates that removal of the sialic acid residues from most glycoproteins results in a greatly shortened plasma half life and the proteins are rapidly taken up into the parenchymal cells of the liver (Morell et al 1971). However this seems unlikely to be of major physiological importance as under normal circumstances the liver seems to be an unimportant site of plasma protein catabolism (McFarlane, 1969; Tavill 1972). Structually abnormal plasma proteins may be formed in certain disease states and such subtle changes may alter the survival in the circulation. Examples are the production of apocaeruloplasmin which is rapidly cleared from the circulation in experimental copper deficiency (Holtzman and Gaumnitz, 1970b) and an abnormal plasma orosomucoid in some

patients with malignant disease (Rudman et al, 1972).

The biological half life of a protein in the plasma is inversely proportional to the fractional catabolic rate which is the amount of protein catabolised expressed as a percentage of the plasma pool mass per unit time. For some proteins like fibrinogen (Freeman, 1967) the fractional catabolic rate is constant regardless of the plasma concentration or pool size. A degree of compensation is built into the system for a fall in plasma concentration is accompanied by a reduction in the total amount of protein catabolised. However in chronic liver disease the fractional catabolic rate may increase due possibly to intravascular coagulation (Tytgat et al 1971). For other proteins such as haptoglobin the fractional catabolic rate seems to be inversely proportional to the plasma concentration (Freeman 1964). This means that a constant amount of protein is catabolised daily and the system has no buffering capacity. In contrast the fractional catabolic rate of albumin is directly related to its plasma concentration and this means that the biological half life alters in response to the pool size thus providing a sophisticated method of compensation (Kirsch et al 1968). The factors determining which pattern of catabolic process occurs with any one plasma protein are entirely unknown and the usual relationship between the plasma concentration and fractional catabolic rate may be disturbed in disease states (Waldmann et al 1972).

Other routes of protein loss may be important in patients with liver disease including leakage into the bowel (Takada et al 1970) and ascitic fluid.

Some of the properties and functions of the plasma proteins studied in the present work will now be mentioned briefly. The significance of the pathological changes found in liver disease will be discussed later.

6. Introduction to the proteins studied in the present work

A. Prealbumin (Prealb)

This protein was first isolated by Schultze et al (1956) who noted it was rich in tryptophan and hence it is sometimes called tryptophan-rich-prealbumin. Its molecular weight has been shown to be 54,000 and the molecule splits into 4 subunits in 6M guanidine (Branch et al 1971). The ability of prealbumin to bind thyroxine was first noted in 1958 by Ingbar. There is one high affinity binding site for the thyroid hormones and 3 other weaker binding sites (Nilsson and Peterson, 1971). Although about 30% of plasma thyroxine is bound to prealbumin, 10% by albumin and 60% by thyroxine binding globulin, the molar ratios mean that less than 1% of the prealbumin molecules carry thyroxine (Smith and Goodman 1971). The significance of prealbumin in thyroid hormone metabolism is uncertain but it is not thought to be an important determinant (Braverman et al 1971).

More recently it has been shown that prealbumin plays a part in vitamin A metabolism. Vitamin A as retinol is almost completely bound in 1:1 molar ratio in the serum to a retinol binding protein, which has been isolated and shown to have a molecular weight of 21,000 (Peterson 1971). The great majority of the retinol binding protein is itself bound to prealbumin and the complex with a molecular weight of 85,000 is too large to pass into the glomerular filtrate and it therefore acts to retain vitamin A in the circulation (Smith and Goodman 1971).

B. Alpha-1-antitrypsin (α_1-AT)

Serum has been known to inhibit the proteolytic action of trypsin for 75 years (Camus and Gley, 1897) but α_1-antitrypsin was not identified as the major protein concerned till its isolation in 1962 by Schultze et al. Its molecular weight is 54,000 and it contains 12% carbohydrate. The name is obviously derived from its action in inhibiting trypsin activity

but it also inhibits chymotrypsin, elastase and to a lesser extent plasmin and thrombin (Fagerhol and Laurell, 1970), as well as proteolytic enzymes derived from polymorphonuclear leucocytes and macrophages (Kueppers and Bearn, 1966). This latter property may be of great importance in understanding the clinical manifestations associated with severely reduced concentrations of this protein in the plasma. Two major types of clinical disease are associated with deficiency of α_1-antitrypsin. A relationship with chronic lung disease was first noted by Laurell and Eriksson (1963) who found 3 cases of emphysema among 5 patients with low plasma levels of α_1-antitrypsin. Since this time many further reports have appeared confirming the relationship (Jones and Thomas, 1971; Hutchison et al, 1972) in up to 29% of patients with severe emphysema. This high figure is a reflection of patient selection. Sharp et al (1969) were the first to point out the association between severe deficiency of α_1-AT and cirrhosis in childhood although a previous report had noted cirrhosis at autopsy in adults dying with emphysema (Ganrot et al, 1967). The role of this protein in the causation of liver disease had not been clearly defined at the time when the present studies were started.

The level of α_1-antitrypsin in the plasma is under genetic control and the data is consistent with multiple codominant alleles at a single locus (Fagerhol and Laurell, 1970). Each allele has been assigned an alphabetical letter on the basis of the electrophoretic mobility of the variant it determines and the system is known as the Pi (proteinase inhibitor) system. Twenty one phenotypes have been described so far using acid starch gel electrophoresis combined with antigen antibody crossed immunoelectrophoresis (Adamson et al, 1971). Most individuals are homozygous for Pi^M (phenotype MM) but up to 13% carry variant genes. (P.Cook, Personal communication). Most of these variant gene products occur in

serum at the same concentration as the product of Pi^M but lower concentrations occur with Pi alleles Pi^P, Pi^S, Pi^W and Pi^Z. By far the commonest is Pi^Z and the homozygous state (ZZ) is associated with the lowest plasma α_1-antitrypsin concentrations and can be identified by simple quantitative techniques. The other phenotypes can be expressed in a range of serum concentrations of α_1-antitrypsin and can be identified only by acid starch gel electrophoresis. The gene frequency varies among different populations but in Great Britain the frequency of homozygous deficiency (ZZ) is about 0.03% and 'heterozygous' deficiency mainly MZ and SZ 2-3% (P. Cook, personal communication). Whether or not patients with intermediate deficiency (usually heterozygous for Pi^Z) are susceptible to lung or liver disease remains undecided as the populations studied in detail so far have not been randomly selected. (Hutchison et al 1972).

C. α_2-Group Component (GC)

was first described by Hirschfeld (1959) who noted an unknown α_2 globulin with 3 different precipitation patterns on immunoelectrophoresis of human sera. The 3 phenotypes were later shown to be under genetic control (Cleve and Bearn, 1962) and the protein was isolated (Cleve et al 1963) and found to have a molecular weight of 50,800 and a 3.3% carbohydrate content. Recent work suggests a single amino acid chain structure (Kuusela and Pihko 1972). The function of this protein is unknown at present.

D. α_2-Macroglobulin (α_2M)

is the major component of the α_2 region on electrophoresis. The protein was first isolated by Brown, et al (1954) and has a molecular weight of about 650,000. It binds and inhibits the proteolytic activity of a variety of proteases including trypsin and chymotrypsin (Saunders et al (1971), plasmin and thrombin (Ganrot 1967)

and kallikrein (Harpel 1970). Thrombin and plasmin have been shown to compete for the same binding site (Ganrot and Nilehn, 1967) and it has been suggested that $\alpha_2$macroglobulin may therefore play a central role in maintaining the balance between blood coagulation and fibrinolysis (Rinderknecht and Geokas, 1972). The action of $\alpha_2$macroglobulin in inhibiting the kinin system may also be important in this respect.

On immunoelectrophoresis the precipitation arc is often asymmetrical suggesting microheterogeneity (Ganrot and Laurell, 1966) and recent studies using polyacrylamide electrophoresis of pure protein at pH 7.8 showed 5 bands, the fastest band being a complex with trypsin (Saunders et al, 1971).

E. Caeruloplasmin (Caer)

is an $\alpha_2$globulin which binds 90-95% of the serum copper. It was isolated in 1948 by Holmberg and Laurell who found the molecular weight to be 151,000 and the copper content 0.34%. The calculated copper content is therefore 8 atoms per molecule. Human caeruloplasmin has also been found to have multiple binding sites for divalent transition metal ions such as zinc and nickel but the physiological significance of this is unknown at present (McKee and Frieden, 1971). Simons and Bearn (1969) have shown that human caeruloplasmin is composed of two different polypeptide chains, α and β, and that the β chain is heterogeneous. There is some evidence that the protein exists in two forms which can be separated from each other by chromatography on hydroxyapatite columns (Trip and van Bruggen, 1972) but the physico-chemical differences between these fractions are presently disputed.

The function of the protein in the body is not clear but it may be important in controlling the liver content of copper. After absorption copper is bound to albumin, is then rapidly cleared by the liver and reappears in the circulation within a few hours incorporated into

caeruloplasmin (Gibbs and Walshe, 1971). High liver copper concentrations are an invariable finding in untreated Wilson's disease in which plasma caeruloplasmin levels are characteristically low. The pathogenesis of the disease and the precise part played by caeruloplasmin are, however, still uncertain (Gibbs and Walshe, 1971). Copper is an essential component of various enzyme systems including cytochrome C oxidase and tyrosinase and caeruloplasmin may be of importance as a copper donor to the tissues. Recently a further role for caeruloplasmin has been suggested by studies in copper deficient animals which have very low levels of caeruloplasmin (Roeser et al, 1970). These animals develop iron deficiency anaemia despite the presence of abundant iron in the tissues, due to an inability to release iron from storage. Caeruloplasmin increases the rate of oxidation of ferrous iron by molecular oxygen and this promotes iron mobilisation from the cell as the binding of ferric iron by transferrin is more avid. The anaemia is rapidly corrected by an intravenous infusion of caeruloplasmin. This function of caeruloplasmin seems to be rate limiting only at extremely low plasma caeruloplasmin concentrations (< 5% normal) but unexplained iron deficiency was found in 5 out of 6 patients with Wilson's disease whose serum caeruloplasmin concentration was less than 5% normal (Roeser, et al 1970).

F. Haptoglobin (Hpt)

makes up about 25% of the α_2 fraction seen on serum electrophoresis. It was first described by Polonovski and Jayle (1938) who noted that the combination of haemoglobin with a plasma constituent increased the peroxidase activity of the haemoglobin. Polymorphism of the protein was noted by Smithies (1955) using starch gel electrophoresis and this is due to the variable structure and combination of the 2 alpha and 2 beta amino acid chains which make up the protein. The Hpα locus is polymorphic with 5 known alleles. Haptoglobin type 1-1 consists of 2 alpha

and 2 beta chains ($\alpha_2^1\beta_2$) with a molecular weight of about 100,000. The Hpα^2 allele causes polymerisation resulting in a series of different molecules (Giblett 1968). 15 phenotypes have been described so far (Braun 1971).

The main physiological function of haptoglobin is to combine in a 1:1 molar ratio with haemoglobin released into the circulation from red cells. There are 4 binding sites on each haptoglobin molecule, 2 for each haemoglobin dimer (Nagel and Gibson, 1971). This complex formation prevents haemoglobin escaping through the glomerulus and may serve to conserve iron as well as prevent renal tubular injury when there is excess intravascular haemolysis. The haemoglobin binding capacity of plasma is 50-150mg./100ml (Nyman 1959) which is at least a hundredfold greater than the normal circulating plasma haemoglobin.

G. Haemopexin (Hpx)

The presence of a haem binding β globulin was first noted in electrophoretic studies by Neale et al (1958) and it was identified immunologically by Grabar et al (1960) who suggested the name haemopexin. Its molecular weight is 57,000 (Seery et al 1972) and it binds haem in a 1:1 molar ratio (Heide et al 1964). Albumin also binds haem (Fairley 1941) the complex usually being known as methaemalbumin, and this is the form in which most plasma haem exists in haemolytic states. However the affinity of haemopexin for haem is greater than albumin (Heide et al 1964; Muller-Eberhard and Liem 1968) and haem is transferred to haemopexin in vitro and in vivo (Muller Eberhard et al 1969) when binding sites become available. Haemopexin also binds a variety of porphyrins (Koskelo et al 1970; Morgan and Muller-Eberhard 1972).

The role of haemopexin in the metabolism of haem has been extensively investigated in the past few years mainly by Muller-Eberhard and her colleagues (Muller Eberhard 1970; Braun, 1971). When haemoglobin is

released into the circulation it is bound to haptoglobin and cleared from the circulation (see above). If the plasma haptoglobin is depleted, some of the unbound haemoglobin dissociates into haem and globin, the iron now being in the ferric form, and this haem binds to haemopexin and albumin. Studies with haem injections in experimental animals and man (Sears and Huser, 1966; Muller-Eberhard et al 1969a; Sears, 1969; Sears, 1970) show that the haem after binding to albumin and haemopexin is rapidly cleared from the circulation with a biological half life of 7-8 hours and the plasma levels of haemopexin fall. Most of the radioactivity is found in the liver and reutilisation of haem iron begins immediately. In the presence of haemopexin depletion more of the haem binds to albumin and the clearance is much slower (Sears, 1970). This is explained by studies with ^{131}I labelled albumin which show that the biological half life is unaltered by haem binding (Muller-Eberhard et al, 1969a) and it seems most likely that albumin is acting merely as a reservoir and transfers the haem on to available haemopexin molecules. Recent studies of the liver uptake of radioactive labelled haemopexin and albumin with varying haem loads confirm the haem reservoir role for albumin (Liem and Muller-Eberhard, 1972).

Autoradiographic studies of rat and rabbit liver at intervals after injection of ^{3}H-haem-haemopexin and ^{125}I-haemopexin-haem complexes showed that all the radioactivity localised in the parenchymal cells (Muller-Eberhard et al, 1970) and this was confirmed recently using an enzymatic method of separating the Kupffer cells from the parenchymal cells (Hershko et al, 1972). There has been great interest in knowing whether both the haem and the haemopexin are catabolised after uptake by the liver. Evidence in favour of haemopexin being a "suicidal" protein like haptoglobin is the depletion of blood levels which occurs with haem injections and the localisation of the labelled protein in the liver. However little fall in

blood levels often occurs despite a large haem load (Sears, 1966, 1970) and this suggested either that the protein turned over very rapidly or that it was merely transporting the haem to the liver and then being returned to the circulation. Recently haemopexin turnover studies in the rabbit (Lane et al, 1972) confirm a rapid biological half life of 23 hours but this may be artefactually fast as the method of isolation of the protein using perchloric acid may have altered some of the characteristics of the protein. Preliminary information on the relative rates of clearance of ^{3}H-haem and ^{125}I haemopexin in the rabbit (Liem and Muller-Eberhard, 1972) suggest that haemopexin recycles back into the circulation after carrying haem to the liver.

H. Transferrin (Trf)

a β globulin of molecular weight 74,000 (Roberts et al, 1966) was first isolated by Surgenor et al (1949). In starch gel electrophoresis 1 of 18 genetically determined slow and fast moving variants can be demonstrated in a small proportion (about 2%) of the population (Bowman, 1968).

The major function of plasma transferrin is to transport iron and each molecule has 2 iron-binding sites, each binding 1 atom of ferric iron. In health the plasma pool of 16 g transferrin (Jarnum and Lassen 1961) is one third saturated and binds about 5mg iron. Most of the iron is taken up by red cell precursors in the marrow and receptors on the red cell precursors facilitate the iron transfer. Studies in vitro with reticulocytes have shown that as their avidity for transferrin is much greater for molecules carrying 2 atoms of iron than 1, the former binding sites are preferentially stripped (Fletcher and Huehns, 1968) and the transferrin, acting as a carrier molecule, is released back into the circulation (Fletcher, 1970). The biological half-life of the iron-transferrin complex

is less than 2 hours and transferrin delivers 30-40mg. iron daily to the marrow for erythropoiesis. The biological half-life of the protein moiety determined by labelling with radio-active isotopes is around 8 days (Katz, 1961; Jarnum and Lassen, 1961; Awai and Brown, 1963) and this agrees well with the survival of unlabelled protein infused into a child with severe congenital transferrin deficiency (Goya, et al 1972).

Recently the role of transferrin in the body's defence mechanisms against infection have been recognised (Bullen, et al 1972). Iron is essential for bacterial growth and there is evidence to suggest that the ability to acquire iron from the host is an essential feature of pathogenicity. Conversely the ability of the host to prevent uptake of iron appears to be an important means of defence. There is now much experimental evidence in vitro and in vivo to support this function of transferrin (Bullen et al, 1972) and in clinical practice the well known susceptibility to infection of malnourished children may be in part due to their very low serum transferrin levels (McFarlane, et al 1970).

A possible role of transferrin in controlling iron absorption has attracted attention for many years. Early studies (Hyde, 1957; Solvell, 1960) had suggested that iron absorption could be increased by raising the circulating level of apotransferrin by infusion and decreased by infusing iron intravenously. However, Wheby and Jones (1963) showed that when the transferrin saturation is increased above about 60% in experimental animals much of the iron is deposited in the liver on the first circulation and examination of peripheral blood samples alone will give misleading information about iron absorption. Using whole body counting techniques these workers showed that raising the saturation of the iron-binding capacity did not reduce iron absorption. More recently Schade et al (1969) in experimental studies in the rat failed to influence iron absorption by

raising the concentration of unsaturated transferrin. A carrier protein for iron in the intestinal mucosal cell has recently been found and although the identity is not yet proven it has certain similarities to transferrin (Hueb et al, 1971; Pollack, et al, 1972). Levine et al (1972) have studied the release of iron from mucosal cells by serum and their results suggest that transport of iron out of the cell maybe aided by the presence of unsaturated transferrin. However in agreement with the findings of Schade et al (1969) they were unable to show that iron absorption was increased in vivo by infusion of unsaturated transferrin. In children with almost complete absence of plasma transferrin iron absorption has been above normal (Heilmeyer, et al 1961; Goya et al 1972). Clearly the part transferrin may play in the transport of iron across the bowel wall is still uncertain.

I. Complement

The 11 plasma proteins which make up the complement system together account for about 10% of the globulin fraction of human serum (Muller-Eberhard, 1969). The third component (C3) is present in greatest amount (100-200mg/100ml.) and is readily identified on immunoelectrophoresis as β_1C globulin. It was first isolated by Muller-Eberhard, et al (1960) and has a molecular weight of around 185,000. Genetically controlled polymorphism is described and 9 phenotypes have been found so far (Alper, 1970).

C3 is converted to C3c in vitro and the electrophoretic mobility changes from β_1C to β_1A. Conversion starts within an hour and is accelerated by coagulation and progresses at a variable rate dependent on the storage conditions (Laurell and Lundh, 1967; Kroll, 1970). Conversion is completely inhibited for up to 12 months by storage at -75°C. (Teisberg 1971) but occurs slowly at higher temperatures. While β_1A is the major in vitro conversion product, Laurell and Lundh (1967) were able to find others using crossed immunoelectrophoresis and a range of conversion

products occurs under different conditions including in vivo activation and treatment in vitro by hydrazine, trypsin or zymosan (Alper, 1970).

Complement participates in antigen antibody reactions leading to cell damage and lysis and apart from its role in defence against infection it is likely that it is involved in a variety of disease states. It is reduced in diseases in which there is evidence of antigen-antibody reactions such as systemic lupus erythematosus and certain types of nephritis (Asherson, 1960; Lancet, 1972) and in these conditions levels of C3 usually run in parallel with total haemolytic complement (Klemperer, et al 1965). On the other hand raised levels are found in a wide variety of inflammatory disorders ranging from myocardial infarction to acute infection (Townes, 1967).

J. Lipoproteins

The plasma lipoproteins can be separated into distinct classes by several methods and the various classifications are based on the methods of separation (Alaupovic, 1972). Ultracentrifugation in media of different densities permits separation into 3 classes, high density (HDL, density 1.063), low density (LDL, density 1.006-1.063) and very low density lipoproteins (VLDL, density less than 1.006). On paper or agarose electrophoresis HDL migrate in the α_1 zone, LDL in the β zone and VLDL in the pre β zone. Ultracentrifugal analysis has shown that VLDL and LDL consist of a population of macromolecules whose molecular weights extend over a continuous range of values. The protein composition of the different ultracentrifugal classes has recently been defined. HDL consists mainly of apolipoprotein A, LDL is 50-60% apolipoprotein B and VLDL is 40-50% apolipoprotein C and 50-60% apolipoprotein B, but all apolipoprotein types are present in each ultracentrifugal class of lipoprotein. Each of the different ultracentrifugal lipoprotein classes contains triglyceride, cholesterol and phospholipid but for VLDL the major lipid component is

triglyceride, for LDL, cholesterol and HDL phospholipid (Levy, et al 1972).

In the present work only lipoproteins with β mobility on electrophoresis are considered.

K. Other Proteins

Other proteins which were regularly identified and measured in the present studies were α_1 easily precipitable glycoprotein (PGP) which was first isolated by Schultze, et al (1963). Nothing is known about its function. Protein 16 originally numbered 45 by Freeman and Smith (1970) may be the same as antichymotrypsin (Aronsen, et al 1972; H.G.M. Clarke, Personal communication). Protein 18 was originally numbered 41 and protein 10 numbered 9 by Freeman and Smith (1970). Nothing is known about the properties or functions of these proteins at present.

7. Introduction to the present work

Abnormalities in the concentration of several plasma proteins have been described in liver diseases. Some like the deficiency of caeruloplasmin in Wilson's disease are of established diagnostic importance but the significance of other changes is uncertain. The development of a method of quantitative immunoelectrophoresis by Clarke and Freeman (1966, 1968) allowed for the first time a large number of individual proteins to be measured easily on a single serum sample.

The present work was started in 1968 in collaboration with the late Dr. Tristram Freeman by applying his technique to a series of patients with haemochromatosis. This study produced some interesting results and because at that time there were no detailed reports in the literature on the changes in concentration of individual plasma proteins in patients with clearly defined types of liver disease, it was decided to study the plasma protein patterns in 3 further diagnostic groups of patients (cryptogenic

cirrhosis, alcoholic cirrhosis and active chronic hepatitis). A collaborative programme of orthotopic liver transplantation with Professor R.Y. Calne of the University of Cambridge also provided a unique opportunity to study the detailed changes in plasma protein patterns after operation.

The original method of immunoelectrophoresis was costly because of the large amounts of antiserum used and a modification requiring smaller quantities of reagents was developed. This method was then applied to the study of the plasma protein changes in patients with acute viral hepatitis, extrahepatic biliary obstruction and primary biliary cirrhosis. Because of the interest of the Liver Unit in hepatic enzyme induction and its clinical and biochemical consequences, a study was undertaken of the changes in normal subjects during enzyme induction and in highly induced epileptics receiving treatment with anticonvulsants.

The concentration of the proteins in the disease groups were compared with a control population and correlations were sought with the clinical data and routine liver function tests. By studying the pattern of plasma protein disturbance in the different types of liver disease it was hoped to gain insight into some of the factors controlling plasma protein synthesis. The possibility that some of the proteins measured might be of value in differential diagnosis was explored by a computer assisted discriminant analysis technique.

PART 2

MATERIALS and METHODS

MATERIALS AND METHODS

1. Immunoelectrophoresis

Two steps are involved, first simple electrophoresis in agarose gel and then electrophoresis at a right angle driving the separated protein fractions into a bed of antiserum to whole human serum in agarose (Clarke and Freeman, 1968).

The first dimension plate was prepared by mixing 20ml. 2% agarose (melted by heating in a boiling water bath) with 20ml. 0.06M barbitone-HCL buffer at 56°C. (Appendix A). The mixture was poured on to a glass plate (16.3 x 11.8cm.) on a levelling table. These agarose covered plates were kept in a damp airtight box at +4°C. for up to a week before use. The plate was placed over a paper plan and a series of 7 holes 1.5mm. diameter were punched in the gel 20mm. apart at points indicated (Figure 1).

Serum (1 ml.) was added to a standard amount of acetylated albumin (Appendix A) with a trace of bromophenol blue and the holes were carefully filled with 4ul. of the mixture using a micro-burette syringe (Micrometric Instrument Co., Cleveland, Ohio). The plate was then placed in the electrophoresis tank (Medical Biological Instrumentation Ltd. type 260) with the origin holes next to the negative electrode. The tanks were filled with buffer diluted to half strength (0.03M) and fresh solution was used each day. Lint wicks were applied with the mat surfaces lying on the gel. A potential difference of 12V/cm. was applied across the plate for about 60 minutes using a Medical Biological Instrumentation Ltd. power supply type 64. The exact length of time of the run was determined by the position of the visible bromophenol blue tagged albumin. The platform of the electrophoresis tank was cooled by circulating water and kept at about 4°C.

Preparations for the second dimension were started immediately so that the time interval between the two electrophoretic steps during which immunodiffusion could occur, was kept as short as possible. A mixture consisting of

2% Molten agarose 9ml. and
0.06M Barbitone-HCL buffer 8ml. at 56°C.

was prepared for each plate and antiserum to whole human serum was added immediately before the plates were poured. The antiserum was raised in goats (Appendix A) and the precise volume added depended on the batch. At the end of the first dimension the plate was placed over the paper grid (Fig.1) and the gel was cut with a long razor blade so as to isolate 7 strips each containing one serum sample. Each strip was then transferred on to the edge of a glass plate 11.6 x 11.6cm. on the levelling table and the plate was completed by pouring on the agarose-buffer-antiserum mixture. Once the antibody containing gel had set, the plates were transferred back to the electrophoretic chamber and fresh wicks were attached. The direction of current flow was reversed to minimise pH change in the buffer and a potential difference of 2.5 to 3 volts/cm. was applied at right angles to the first run for excess time (18-24hrs.) driving the protein into the bed of antiserum. No cooling was required.

After the second dimension run the slides were washed in physiological saline for 24hrs. and then in deionised water for 30 minutes. They were wrapped in Whatman No.1 filter paper and dried at 37°C. before staining.

2. Modifications of the Method for use with Miniplates (5 x 5cm.)

The agarose and buffer used were made up in the same way as for the large plates. As the goat antiserum was no longer available, commercially available antiserum to whole human serum raised in sheep was used (Paynes and Byrne Ltd. Greenford, Essex) and the same batch (No. 551019) was used throughout. Acetylated albumin (Paynes and Byrne Ltd.) was used as a reference protein and this was made up in ampoules containing a fixed quantity to which was added 0.5ml. of test or standard serum. A different batch of acetylated albumin

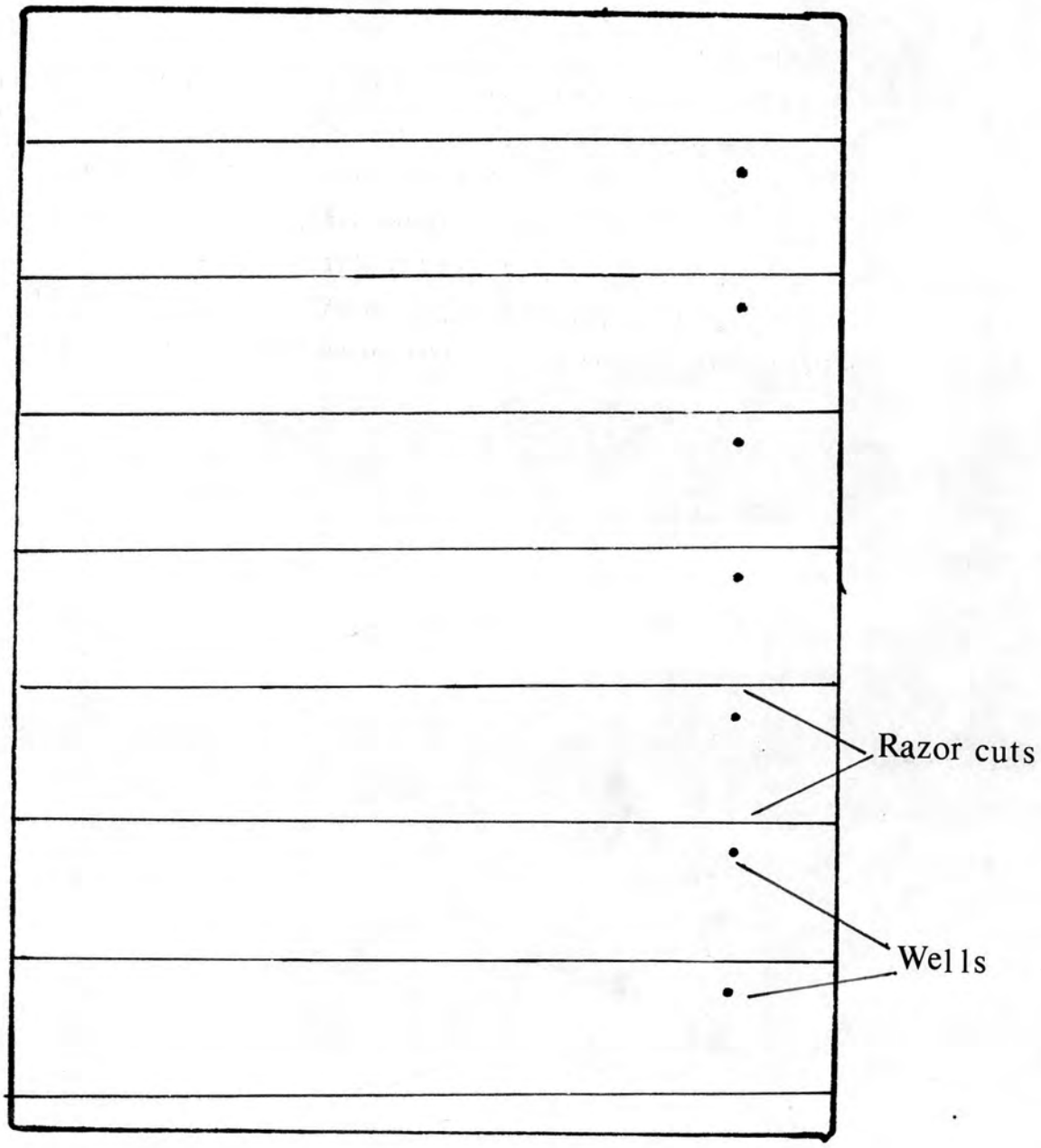


Figure 1. Plan showing the position of the seven wells and the lines for the razor cuts to separate the agarose strips for the second dimension run.

was used for some studies and to this was added a trace of bromophenol blue and 0.2ml. of test or standard serum. Freeze dried standard serum (batch no. 551019 Paynes and Byrne Ltd.) reconstituted with 1ml. deionised water per ampoule was used throughout.

The first dimension plate was prepared by mixing

2% Molten agarose 2.6ml.
0.06M barbitone-HCL buffer at 56°C. 2.6ml

This was poured on to a level glass plate 5 x 5cm. and allowed to cool. The plate was placed over the paper plan (Fig.2) and 4 holes 1.5mm. diameter were punched in the gel and filled with approximately 1µl. of the acetylated albumin and serum mixture using a microcap pipette (Drummond Scientific Company). The first dimension was run at 7V/cm. without cooling until the bromophenol blue marked albumin had travelled about 20mm from the front of the origin hole. The gel was then cut as before and after transfer of the first dimension strips to clean 5 x 5cm. slides the second dimension plates were prepared by pouring on a mixture containing

2% agarose 1.9ml
0.06M barbitone buffer 1.8ml
Antiserum 0.13ml.

The plates were stacked in piles of 3 on top of one another (Fig.3) and all 12 samples from the first dimension were accommodated in the same tank. The current was reversed and a potential gradient of 1V/cm. was applied at right angles to the first dimension and the electrophoresis run for 16-20hrs (overnight). The 12 plates were placed in a projector slide carrier (Kodak) and washed in physiological saline at 37°C. for 30-60 minutes and then deionized water for 5-10 minutes. They were then dried as before.

3. Staining

Plates were routinely stained with Amido black (Appendix A) for 5-10 minutes. The background stain was then removed with alcohol-acetic wash (Appendix A), rinsed in methanol and dried (Frontispiece and Fig. 4).

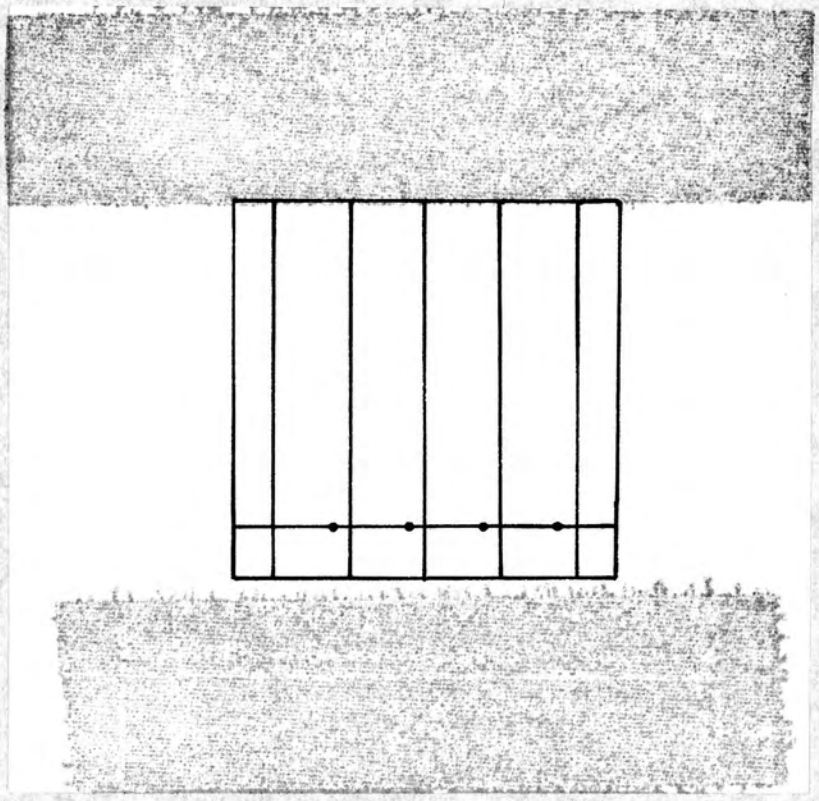


Figure 2. Plan showing four wells and the lines for the razor cuts to separate the agarose strips for the second dimension run on the mini plates.

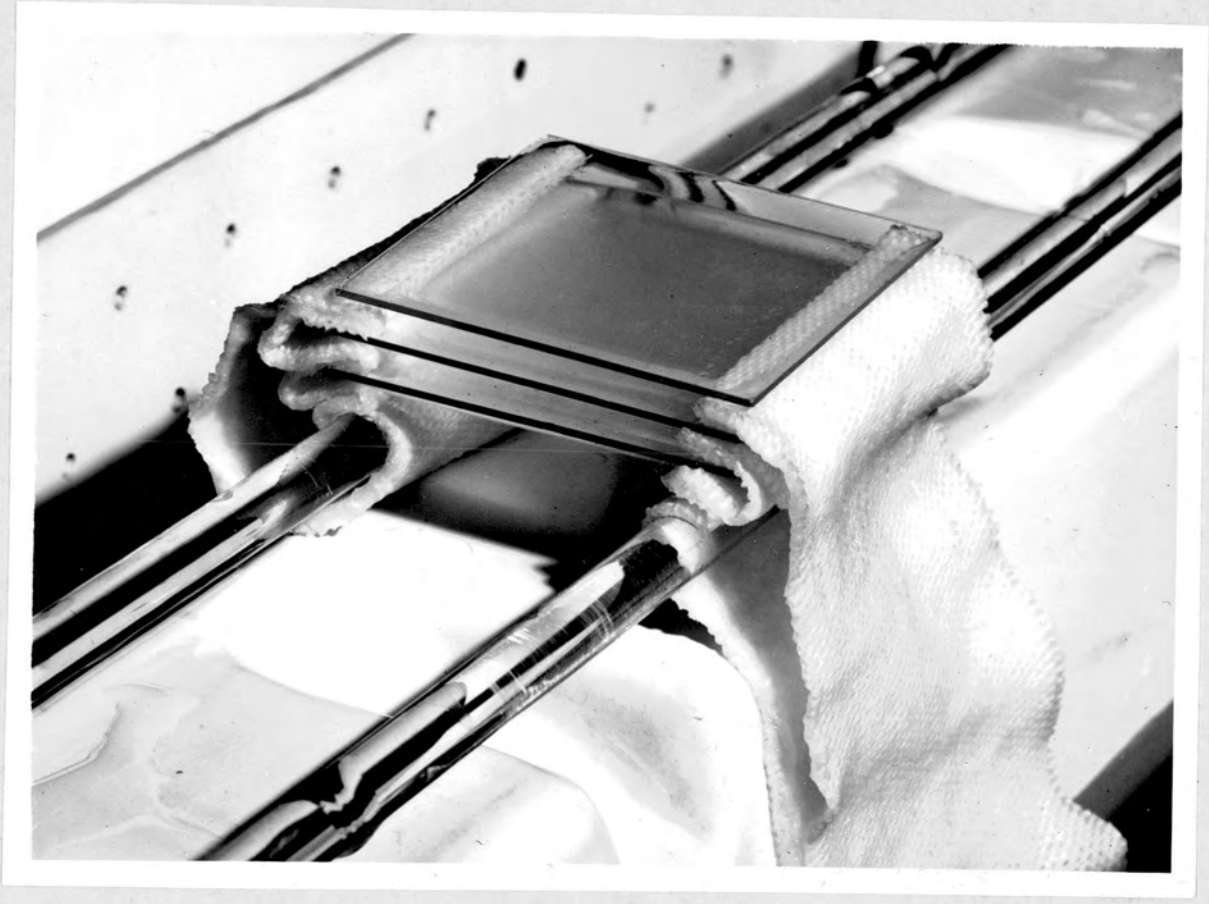


Figure 3. Three plates stacked on top of each other for the second dimension run showing the arrangement of the lint wicks.

4. Identification of Individual Proteins

Initially as many proteins as possible were identified by immunological methods and special staining. Many proteins were readily recognised in all sera by their characteristic electrophoretic position, distinctive shape of precipitation arc and avidity for stain. In pathological sera the relative heights and positions of the arcs were often altered and it was necessary to run each sample several times so that different specific staining methods could be applied.

A. Specific Staining of Individual Proteins

i) Caeruloplasmin

The plate was soaked for 15-20 minutes at 37°C. in an 0.08% solution of ferrous sulphate in 0.1M acetate buffer pH 5.7 (Appendix A). It was washed in deionized water, dried and stained for about 1 minute in a mixture of equal volumes 2% potassium ferrocyanide and 0.2N HCl. Caeruloplasmin turned blue and the plate was counterstained with 0.2% solution Ponceau red in 5% Trichloracetic acid (Fig. 5).

ii) Haptoglobin and Haemopexin

These proteins stain only when binding haemoglobin or haem respectively and staining can be intensified by adding a trace of haemolysed serum to the sample. 100mg. benzidine was dissolved in 50ml. boiling deionized water and while still hot 0.25ml. glacial acetic acid was added. The solution was then cooled and 0.1ml. 30% hydrogen peroxide added. Plates were stained in this solution for 20-30 minutes and haptoglobin and haemopexin stained black (Fig. 6).

iii) Lipoproteins

The plate was stained for 2 hours in 0.5% solution Oil red O in 50% ethanol and then washed in 50% ethanol. Lipoproteins stain red. Alternatively Sudan Black was used (Appendix A). The plate was soaked for 24 hrs. and then washed in 50% ethanol. Lipoproteins stain black (Fig. 7).

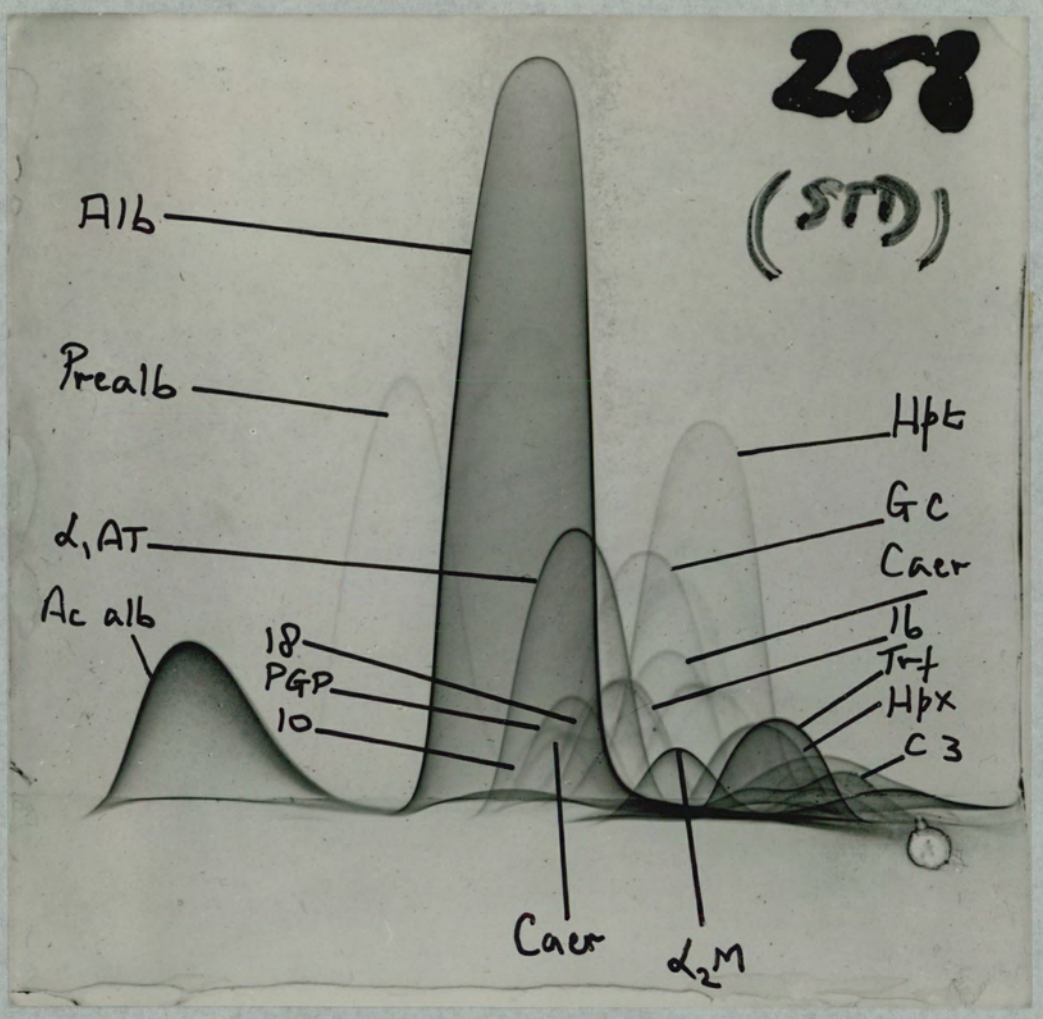


Figure 4. Paines and Byrne standard serum run on mini plate and stained with amido black.

Figure 5. Plate stained for caeruloplasmin (blue) and counter-stained with Ponceau red.

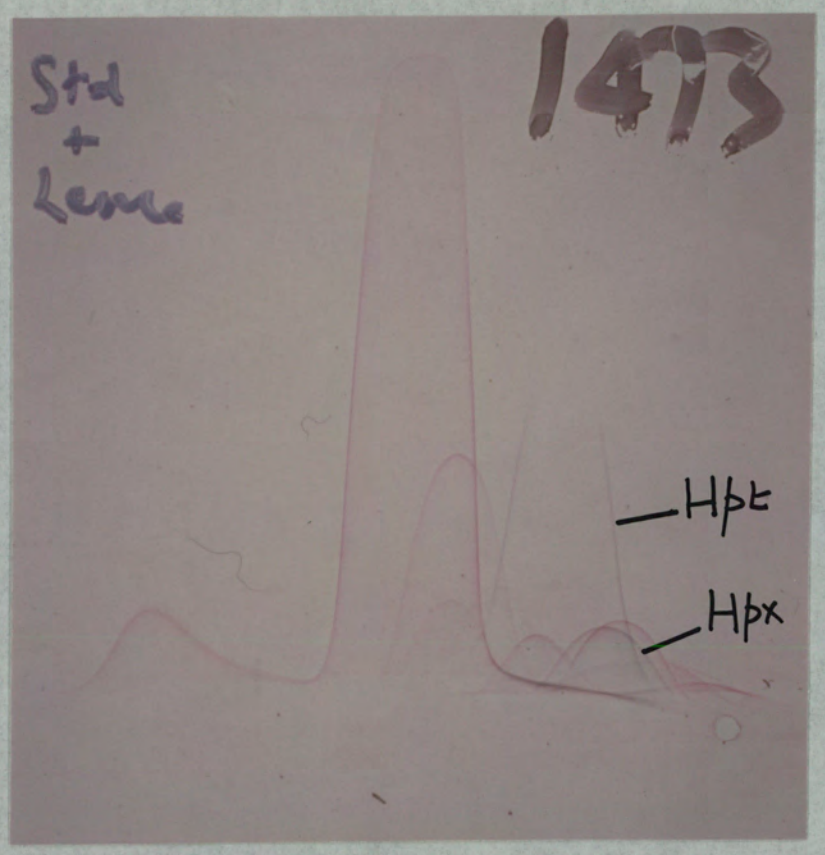


Figure 6. Plate stained for haptoglobin and haemopexin (black) and counterstained with Ponceau red.

Figure 7. Plate stained with Sudan black to show β-lipoprotein.

An alpha lipoprotein precipitation arc developed with the goat antihuman serum used in the early studies but the sheep antiserum used for the miniplates did not contain a significant titre of antibodies to α_1 lipoprotein and no precipitation arc formed. β lipoprotein is destroyed by freeze drying and did not therefore appear in the Paynes and Byrne standard serum (Fig. 4) but it was readily identified in all other samples (Fig. 7).

B. Identification using pure Protein

Transferrin was identified by placing pure protein (Hoechst Pharmaceuticals) in a second origin well. The protein fused with and distorted the contour of the transferrin arc in the test serum (Fig. 8 and 9).

C. Identification using Monospecific Antisera

α_2 group component, α_2 macroglobulin and α_1-antitrypsin were identified by this method. An equal volume of monospecific antiserum (Hoechst Pharmaceuticals) was placed with the test serum in the origin well and the specific protein was identified by reduction in area under the precipitation arc. Other protein areas were either unaltered or increased in area due to the adsorption procedure used to prepare the antiserum (Fig. 10). Confirmation of identification came from the characteristic shape of the α_2 group component peak in infants (Abrams and Freeman 1969) and the marked reduction of the α_1 antitrypsin peak in sera from patients known to be homozygous deficient subjects (Fig. 11).

D. Other Methods

Tryptophan rich prealbumin is the only protein migrating faster than albumin at pH 8.6. The small flat curve seen at the cathodal end of the Paynes and Byrne acetylated albumin peak is a contaminant as it appeared when this protein alone was run (Fig. 12). Proteins which have been

Figure 8. Pure transferrin run against antiwhole human serum showing single precipitation arc.

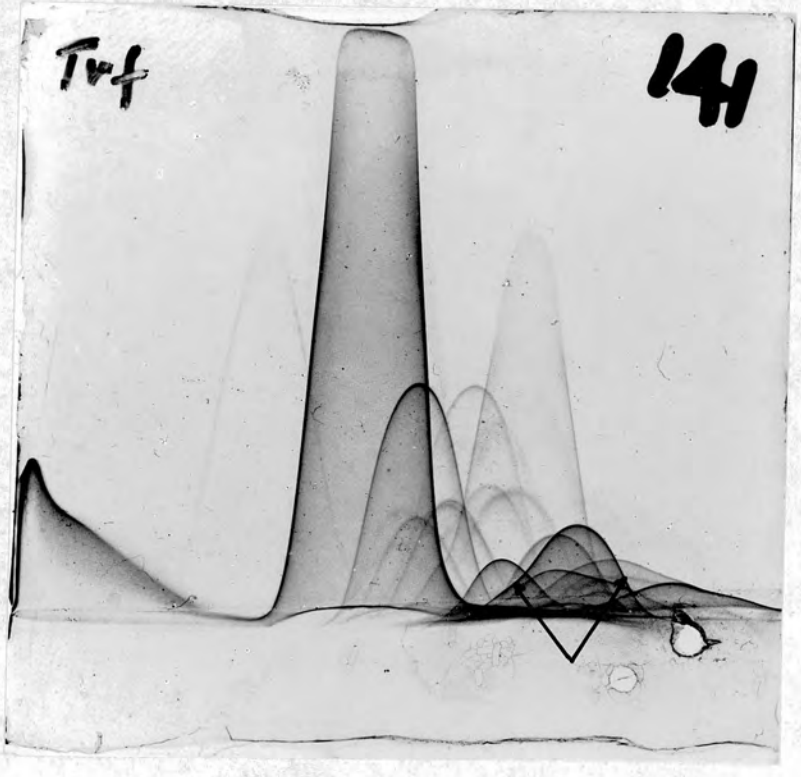


Figure 9. Pure transferrin in second origin hole producing distortion of transferrin peak in standard serum.

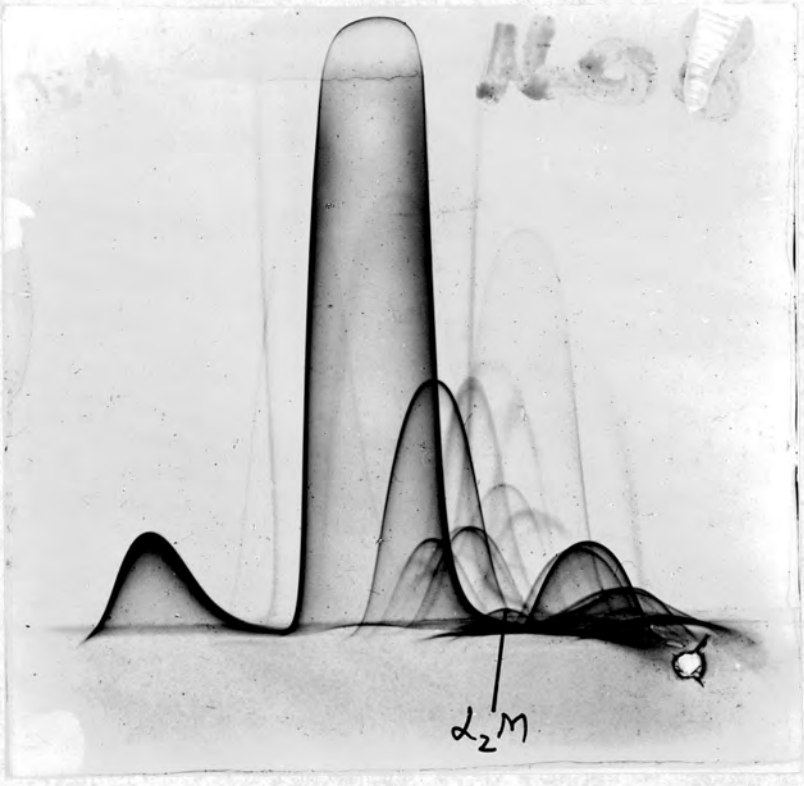


Figure 10. Anti α_2-macroglobulin antiserum added to standard serum in origin hole. Compare α_2M peak with figure 4.

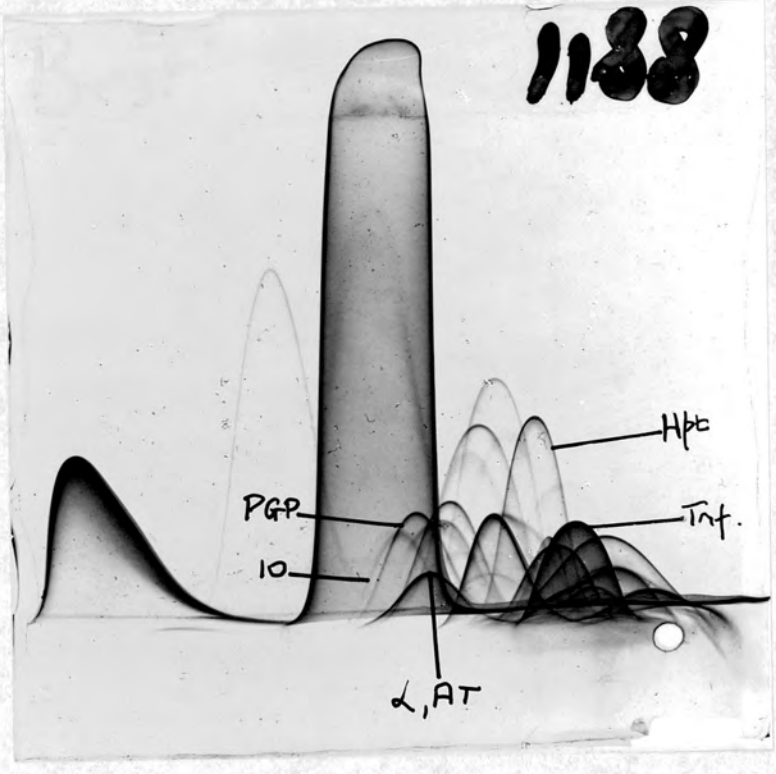


Figure 11. Serum from patient with severe deficiency of α_1-antitrypsin.

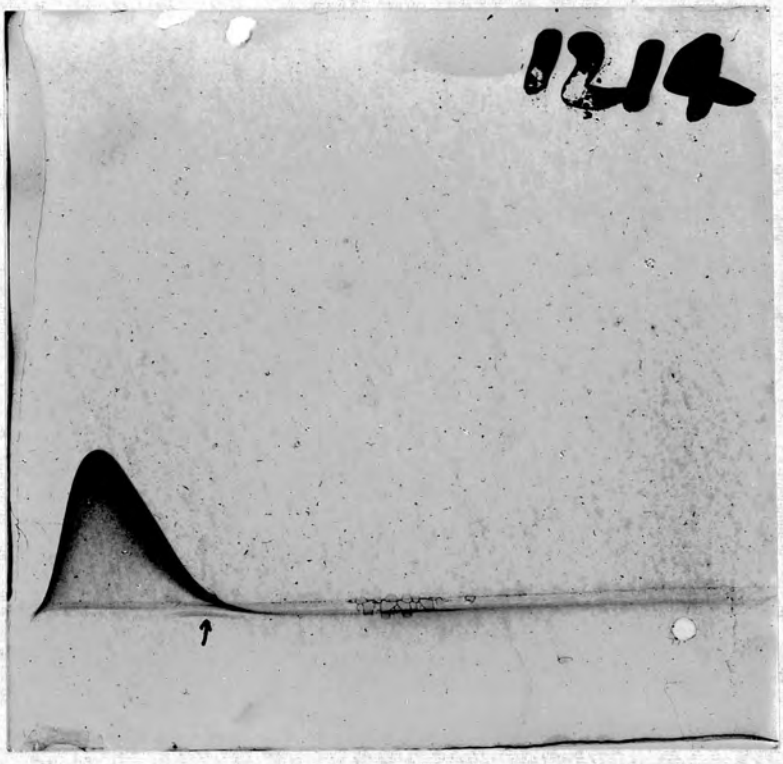


Figure 12. Acetylated albumin run with antiserum to whole human serum. Note small arc at the cathodal foot of the acetylated albumin.

provisionally identified in the α_1 band and which were regularly measured in normal and pathological sera were α_1-easily precipitable glycoprotein, proteins 10, 16 and 18. These last three were originally numbered 9, 45 and 41 respectively (Freeman and Smith, 1970). β_1 A-C (C3) was identified because of the characteristic double contour and increasing proportion of the faster moving component (β_1A) on storage (Fig. 4). As the arc was small and difficult to identify on the mini plates it was not measured.

Numerous other precipitation arcs were seen but not positively identified. Many were faint and none was sufficiently constant in position for measurements to be reliable.

5. ARTEFACTS

Two artefacts were encountered from time to time.

A. Doubling. (Fig. 13). The precipitation arc in the second dimension is not perpendicular to the surface of the gel and after drying this results in broadening or doubling of the line. This effect may be due to failure to cut the first dimension gel at a right angle to its surface or to drying of the gel surface in the first dimension. Duplicate measurements on separate electrophoretic runs of the same sample showed that valid results could be obtained by taking the mean of the areas enclosed by the two lines. Certain protein arcs (α2 macroglobulin and β lipoprotein) were frequently doubled when all other protein precipitates on that plate were satisfactory.

B. Trailing . Albumin sometimes failed to migrate uniformly but left a 'trail' leading back to the origin hole (Fig. 14). The precipitation arcs of other proteins were seldom disturbed and measurement on standard serum with and without albumin trailing showed no difference in the values for other proteins.

Figure 13. Plate showing doubling artefact.

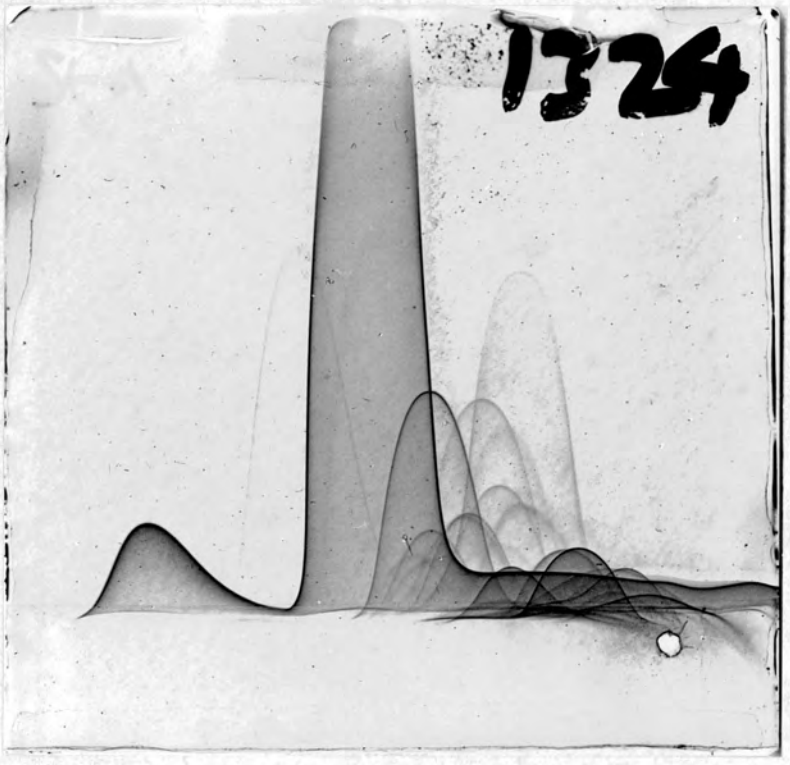


Figure 14. Plate showing 'trailing' of albumin to the origin.

6. Measurement of Protein Areas

The plate was placed in a photographic enlarger and the areas under the peaks were measured using an electronic integrator designed by J. Lewin of the National Institute for Medical Research and manufactured by Chemical Electronics Co. (C.W.S. Hall, Durham Road, Birtley, Co. Durham). The base line for all proteins except acetylated albumin was set from the cathodal foot of the transferrin peak to the anodal foot of the albumin peak (line A, Fig. 15) and was reset to measure the acetylated albumin area (line B, Fig. 15). At least 3 measurements were made for each protein and the mean calculated. The data were recorded on a laboratory work sheet (Appendix B).

7. Calculation of Results

The use of the acetylated albumin as an internal standard obviates the necessity for accurate pipetting of microlitre quantities of serum. As the same volume of serum was added on each occasion to the acetylated albumin ampoule, it follows that the ratio $\frac{\text{area of unknown protein}}{\text{area of acetylated albumin}}$ is constant and reproducible for any serum sample. This ratio was then related to the ratio obtained for the same protein in a standard serum

$$\frac{\text{Area of unknown protein (Y)}}{\text{Area of acetylated albumin on unknown plate}} \times \frac{\text{Area of acetylated albumin in standard serum}}{\text{Area of protein Y in standard serum}} \times 100$$

The unknown protein was thus expressed as a percentage of concentration of the same protein in the standard serum. The value for the standard used in the above calculation was taken as the mean of the readings on 20 standard plates. A standard serum sample was included in most electrophoretic runs and if the area for each protein fell out with the normal range established from 20 standard plates run at different times, the whole run was discarded.

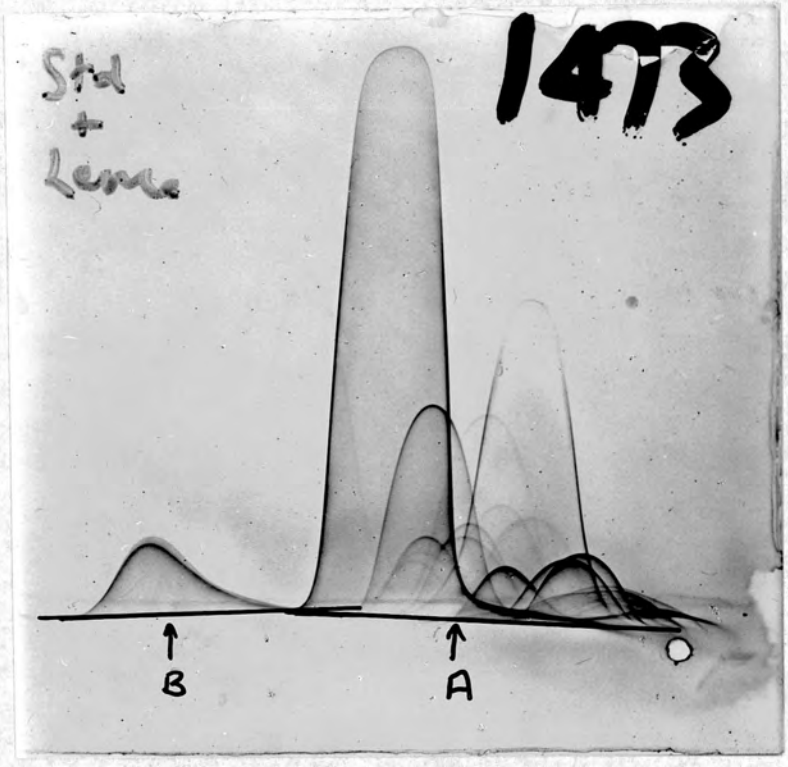


Figure 15. Baseline for measuring plasma proteins (line A) and for acetylated albumin (line B).

8. Standard Sera

For the early part of this work on large plates a freeze dried reference serum was obtained from the Department of Biological Standards, National Institute for Medical Research, Mill Hill (Code No. 67/86). It was derived from a pool of 416 male blood donors. A sample of this standard serum which had been stored at -20°C for 4 years and -70°C for 1 year was later run with Paynes and Byrne antiserum on mini plates (Table 1).

The second freeze dried standard (Paynes and Byrne Ltd.) used in the miniplate studies was derived from a pool of 20 male and female donors and this contained no β-lipoprotein (See above).

A third standard serum (Hoechst Pharmaceuticals Batch no. 570) was also studied and by comparing the areas of certain proteins with those of the same proteins in the Payhes and Byrne standard serum, absolute values for these were derived.

9. Other Methods

A. Complement Studies using monospecific antiserum for Immunoelectrophoresis

Blood was taken into potassium edetate bottles and plain glass containers and spun within 15 minutes. The immunoelectrophoretic technique was modified so that 2.5ul. of the fresh serum and plasma from the same patient were run one in front of the other on the same first dimension strip. The second dimension plate was made up in the usual manner but using 0.05ml. antiserum to β_1A-C (Hoechst Pharmaceuticals).

B. Radial immunodiffusion

Haemopexin was measured by radial immunodiffusion on M Partigen plates (Hoechst Pharmaceuticals) which were calibrated by using 4 dilutions of a standard serum (Hoechst Pharmaceuticals) with a known concentration

Table 1

Protein concentrations in National Institute for Medical Research serum pool (No. 67/86). Mean value of 8 determinations expressed as % of acetylated albumin marker and % Paynes and Byrne standard serum.

Protein	Mean Area	S.D.	% Paynes and Byrne standard serum
Prealb	290.9	5.1	85.0
α_1PGP	55.4	3.2	86.5
α_1AT	164.6	4.2	81.9
GC	174.6	6.9	84.6
α_2M	32.3	2.1	91.3
Caer	104.4	3.9	98.5
10	35.5	3.4	89.9
Hpt	336.0	26.8	90.5
Hpx	49.5	3.5	82.4
Trf	47.8	4.4	77.8
βLP	19.6	1.3	-
16	67.6	4.0	81.6
18	78.0	2.6	92.8

of haemopexin. The diameter of the precipitin rings was measured after 48hrs. and the squares of the diameter of the standard serum and its dilutions were plotted on a linear scale. The concentration of haemopexin in the unknown samples was then read off the straight line calibration graph.

C. Plasma bilirubin levels for the enzyme induction study were measured by Thompson's modification of Michaelsson's method (1969).

D. Urinary D-glucaric acid output was measured by the method of Hunter et al (1971a).

E. Routine liver function tests were performed in the Department of Chemical Pathology on an SMA 12 Autoanalyzer and protein electrophoresis on cellulose acetate strips.

F. The differential ferrioxamine test was performed according to Fielding (1965).

PART 3

VALIDATION OF THE TECHNIQUE USING MINIPLATES

VALIDATION OF THE TECHNIQUE USING 5 x 5cm. MINIPLATES

1. Multiple Serum Dilutions

As a check that the technique was quantitative using the miniplates and the volumes of serum and antiserum chosen, standard serum was diluted with distilled water to contain 80, 66.6, 50 and 33.3% protein. Ten proteins were measured at each of these dilutions and for each protein there was a good linear correlation between area and serum dilution (Figs. 16 and 17).

2. Errors in repeated measurements of standard serum

A. Repeated measurements of a single plate

i) Twelve proteins were measured on 6 occasions in one day on the same plate without removing it from the photographic enlarger or disturbing the base line setting. (Table 2).

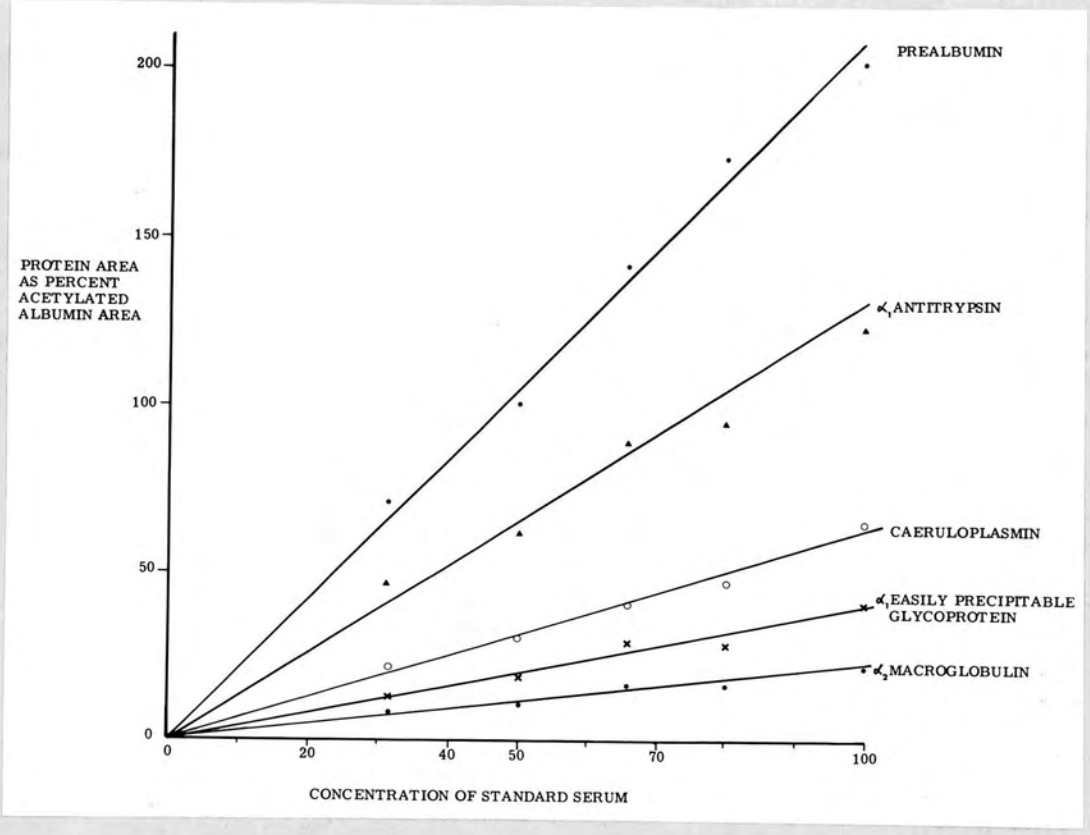
ii) One plate was measured on 8 occasions in one day but it was removed from the enlarger and the base line reset before each series of measurements. This resulted in a larger coefficient of variation in 9 of the 12 proteins measured (Table 2).

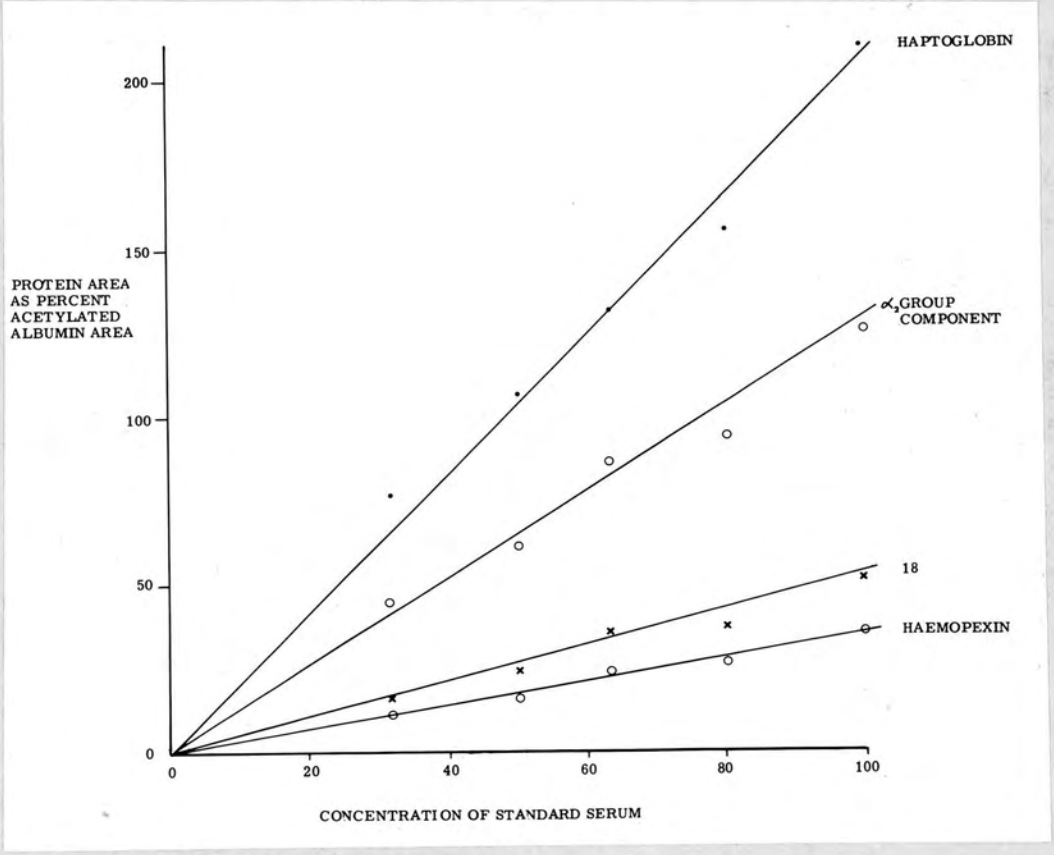
B. Agreement between plates in the same run

Twelve plates, each containing a sample from the same ampoule of standard serum, were run simultaneously and the coefficient of variation (Table 3) for all 12 proteins was larger than when a single plate was measured (section Aii above; Table 2).

C. Agreement between plates in different runs

Twelve plates containing standard serum from different ampoules were run on 12 different days. The coefficient of variation was larger than those obtained in Section 2B for each protein due to the introduction of errors in pipetting





Figures 16 and 17. Correlation between dilution of the standard serum and area under the protein arc expressed as percentage of the acetylated albumin area for nine of the proteins studied.

Table 2

Error in repeated measurement of the area of 12 proteins in standard serum (expressed as % of area of acetylated albumin marker) on a single* 5 x 5cm. plate.

Protein	Baseline unaltered			Baseline reset		
	Mean Area (n=6)	S.D.	Coefficient of variation	Mean Area (n=8)	S.D.	Coefficient of variation
Prealb	292.8	4.6	1.6	191.9	5.2	2.7
α_1PGP	57.0	0.6	1.1	37.4	0.9	2.5
α_1AT	170.8	1.7	1.0	115.2	1.0	1.9
GC	179.5	1.6	0.9	114.0	3.0	2.6
α_2M	34.3	0.5	1.3	21.7	0.8	4.1
Caer	110.7	3.2	3.0	54.5	2.0	3.7
10	38.1	1.6	4.2	22.7	2.2	9.8
Hpt	350.5	2.6	0.8	190.0	4.2	2.2
Hpx	52.2	1.2	2.2	35.2	0.7	2.1
Trf	50.0	1.2	2.5	36.5	0.7	2.0
16	71.6	2.0	2.7	47.8	1.1	2.3
18	80.5	1.4	1.7	49.0	1.4	2.9

* A different plate was used for each of these two experiments

Table 3

Error in measurement of multiple samples of the same standard serum run simultaneously and at different times on 5 x 5cm. plates. Area is expressed as % of area of acetylated albumin marker.

Protein	Same run			Different runs		
	Mean Area (n=12)	S.D.	Coefficient of variation	Mean Area (n=12)	S.D.	Coefficient of variation
Prealb	201.1	11.2	5.6	198.6	16.6	8.4
α_1PGP	39.6	2.4	6.2	39.9	5.5	13.8
α_1AT	121.8	4.3	3.6	122.5	8.6	7.0
GC	124.7	4.7	3.8	124.2	7.7	6.2
α_2M	21.0	1.3	6.4	22.6	2.0	8.8
Caer	63.3	2.6	4.1	57.7	4.5	7.9
10	23.2	2.1	9.1	23.5	4.5	19.1
Hpt	211.0	23.0	10.9	199.0	30.0	15.3
Hpx	36.3	1.3	3.6	37.1	3.7	10.0
Trf	36.5	2.0	5.4	38.8	3.9	10.0
16	49.4	1.79	3.5	47.6	4.8	10.2
18	51.2	1.7	3.4	49.4	4.8	9.7

the standard serum into the acetylated albumin ampoules, differences in the acetylated albumin content of the ampoules and variation in the conditions of the electrophoretic run from day to day.

Because of the variation in the standard serum protein values in any one electrophoretic run, a mean value from 20 runs was used when calculating the concentration of an unknown serum as a percentage of the standard serum.

3. Comparison of immunoelectrophoresis with radial immunodiffusion

Haemopexin was measured in 16 serum samples by radial immunodiffusion and the result expressed in mg/100ml. by reference to Hoechst standard serum. The same serum samples were measured by immunoelectrophoresis on miniplates and there was close agreement (Fig. 18) between the results by the two methods for most samples ($r=0.940$; $t=10.33$; $p < 0.001$).

4. Effects of Storage of Serum

Serum was taken from two normal individuals, divided into 1 ml. aliquots and stored at -20°C. without added preservative. Samples from the store were tested 9 times at intervals over a 12 month period and care was taken that they had not previously been thawed and re-frozen. There was little overall change in the concentration of any of the 12 proteins over the 12 month period with the exception of β lipoprotein which fell in both subjects (by 30 and 50%). The variance of the concentration of each protein was no greater (F Test) than that found on repeated measurements of the standard freeze dried serum and in some instances it was significantly less (Table 4).

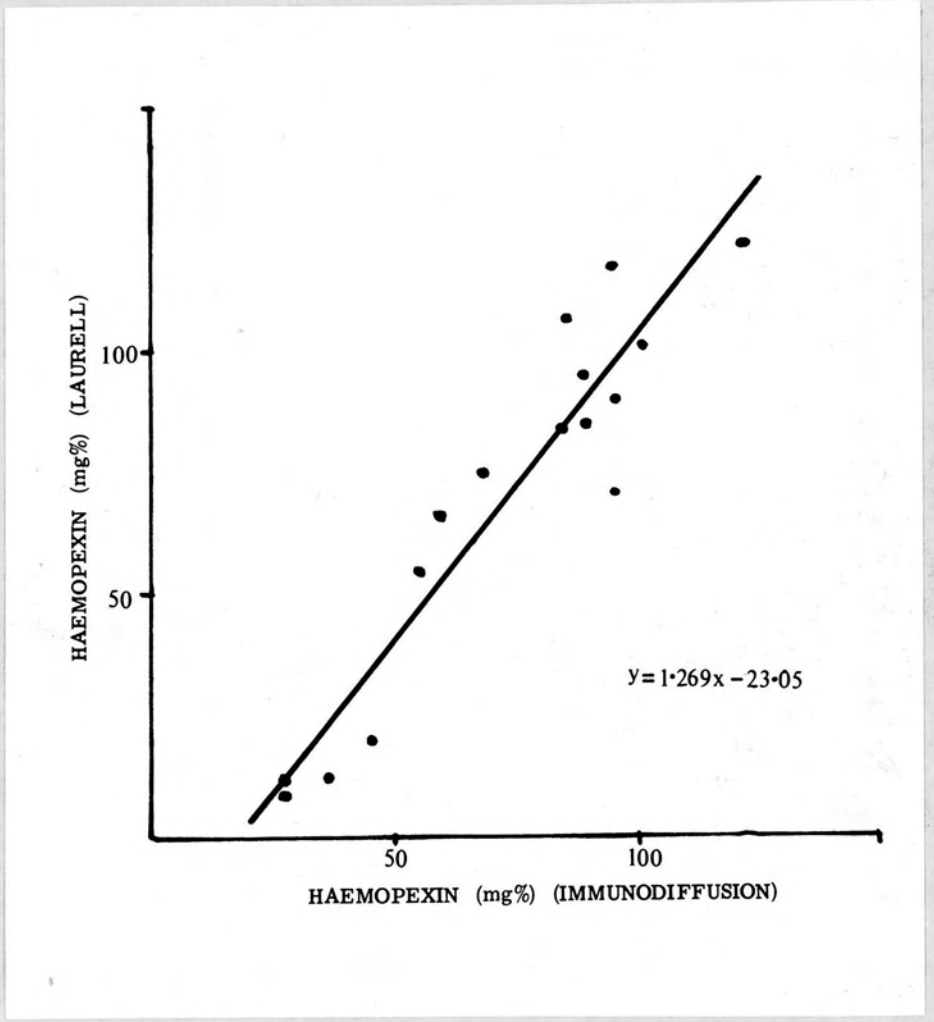


Figure 18. Correlation between haemopexin concentration in the same serum sample measured by radial immunodiffusion and immuno-electrophoresis (Laurell).

Table 4

Repeated measurement over 12 months of stored sera from 2 subjects. Results expressed as % of the acetylated albumin marker and the variance compared with that of repeated measurement of standard serum.

	Subject 1			Standard serum			Subject 2	
Protein	Mean Area (n=9)	S.D.	Pvalue	Mean Area (n=12)	S.D.	Pvalue	Mean Area (n=9)	S.D.
PGP	42.78	1.39	**	39.9	5.5	**	35.44	1.59
α_1AT	111.22	2.73	**	122.5	8.6		117.56	7.76
GC	151.78	8.14		124.2	7.7		120.44	10.17
α_2M	20.56	1.24		22.6	2.0		20.11	3.06
Caer	70.11	1.76	**	57.7	4.5		62.33	1.97
10	35.56	1.88	*	23.5	4.5	*	28.44	2.24
Trf	43.22	2.91		38.8	3.9	*	38.22	2.11
Hpx	39.00	2.74		37.1	3.7		35.33	2.40
16	57.78	2.23	*	47.6	4.8	*	47.78	2.11
18	63.78	2.39	*	49.4	4.8		58.11	3.59

** P $<$ 0.01 * P $<$ 0.05

5. COMMENT

The immunoelectrophoretic method using large plates which was employed in the early studies was costly because of the large volumes of antiserum needed. It was therefore decided to adapt the technique to 5 x 5cm. plates which have the attraction of being suitable for immediate display using a standard slide projector. The thickness of the agarose gel was kept to the minimum consistent with ease of handling, in order to reduce the doubling artefact and volume of antiserum required. The accuracy of the method is greatest when the precipitin lines are faint (Laurell, 1972) and the volumes of the test serum and antiwhole human serum chosen were influenced by this and the need to keep the whole precipitation arc of the proteins to be measured on the plate. It was not possible to measure all proteins on a single plate because some peaks (e.g. albumin, alpha-1-acid glycoprotein (orosomucoid) and alpha-2-HS-glycoprotein) ran off the agarose in the second dimension. These proteins could however be measured by using less serum or more antiserum but then the accuracy of measurement of other proteins was severely reduced because of the small size of their precipitation arcs. This is the only major disadvantage of the miniplates compared with the large plates but it was regularly possible to identify and measure 13 proteins on one electrophoretic run. The voltage in the second dimension was chosen to produce peaked arcs, and as little heating of the gel occurred, cooling was not necessary. Using a lower voltage for a longer time allowed lateral diffusion to occur and the peaks became broad and flat with loss of accuracy in measurement. A similar scaled down modification of the original method was later published by Davies et al (1971) and Stephan and Frahm (1971).

With experience the recognition of the different precipitation arcs presented little difficulty although in pathological sera, in which the concentrations of many proteins were altered, several specific stains

were often used to confirm identification. For some proteins (e.g.easily precipitable glycoprotein, proteins 10, 16 and 18) identification is provisional and based on a detailed fractionation study of human serum using gel filtration combined with immunoelectrophoresis (Freeman and Smith, 1972). As the same pool of antiserum was used for all the miniplate studies, the identification and nomenclature is uniform throughout. It is impossible at present to identify with certainty all the corresponding proteins among those illustrated and numbered by Weeke (1970b) who was using a different antiserum. Because of electroendosmosis and the slight migration of the immunoglobulins at pH 8.6, these cannot be measured accurately by this method, but valid readings are possible after reacting the proteins with potassium cyanate (KCNO) to increase their isoelectric points (Weeke 1968). As β_1A-C produced a small arc which was difficult to identify and measure accurately on the miniplates, no values are given. Storage of sera introduces a further variable error as the value for β_1A-C rises by up to 21% as conversion to β_1A occurs (Kohler and Muller-Eberhard, 1967).

This immunoelectrophoretic method on large plates was shown to be quantitative for each protein measured (Clarke and Freeman, 1966) and this was confirmed in the present study on miniplates by using several serum dilutions and by comparing the results of haemopexin measurements with those obtained by radial immunodiffusion. The errors in the method were explored systematically and the coefficient of variation for most proteins was less than 10% when the same standard serum was examined on different occasions. This compares very favourably with the results on large plates (Clarke and Freeman, 1968). The use of acetylated albumin as an internal standard eliminates the great potential error involved in delivering one microlitre of serum into the first dimension well, but this method obviously depends

on the ampoules containing a fixed quantity of acetylated albumin and accurate pipetting of 1 ml. serum into the ampoule. Other workers have used carbamylated transferrin as a marker protein in the same way (Weeke, 1970). The coefficient of variation for repeated measurements of protein 10 was larger than for other proteins probably because the arc was faint and difficult to measure. The haptoglobin arc varied considerably from plate to plate and others have also found high coefficient of variation in its measurement (Clarke, Personal communication). Because there was considerable variation in protein values between plates of the same serum in the same run, the standard serum values taken for reference were based on the means of 20 plates. A standard serum sample was included subsequently with unknown sera in most runs and the concentration of all proteins in the standard serum usually lay close to the mean value calculated previously from 20 standard plates.

Storage of serum from two subjects for up to 12 months at -20°C. showed a significant fall in the concentration of β lipoprotein but the levels of other proteins were unaltered. There is little information in the literature on the effects of storage on protein concentrations but Cohnen and Paar (1969) found a 15-20% fall in most proteins after 20 months at -28°C. Repeated freezing and thawing of samples is far more damaging and they found marked reduction in all protein levels after 8-10 cycles. Similar results were reported recently by Weeke (1972) who noted a 10-15% fall in most proteins after storage at -18°C. for 40 months. The addition of sodium azide to the serum minimises the changes. The conversion of β_1C to β_1A and the increase in total area has been mentioned and similarly caeruloplasmin changes to faster moving apocaeruloplasmin (Fig. 4). Measurement of both parts overestimates the total concentration and the main peak alone gives the best approximation to the original value (Weeke, 1972). The standard serum used in the large plate studies had been stored

at -20°C. for four years and -70°C. for 1 year before it was measured on miniplates and this may account in part for the values of most proteins being 10-15% lower than those in the standard serum used in the miniplate studies. Weeke (1972a) has shown that plasma pools derived from large numbers of healthy people differ little but the number of donors to the miniplate pool was small (20 subjects) and included both sexes whereas the large plate pool donors were males only. This difference in the standard sera makes it difficult to compare the results from the large plate and miniplate studies which are expressed as a percentage of their respective standard serum pools.

The concentration of plasma proteins in animals undergoes circadian rhythm (Scheving, et al, 1968; Bocci and Viti, 1971) and although there is no information available for man there are likely to be changes because of variation in blood volume particularly in those subjects with splenomegaly (Blendis et al, 1970). Hard physical exercise has also been shown to alter the concentrations of some proteins (Poortmans, 1971). Because many of the patients studied in the present work were out-patients, it was not possible to standardise the timing of blood samples or to ensure that the patients were fasting.

PART 4

DESCRIPTION OF NORMAL SUBJECTS AND PATIENT GROUPS STUDIED; STATISTICAL METHODS

DESCRIPTION OF NORMAL SUBJECTS AND PATIENT GROUPS STUDIED AND STATISTICAL METHODS

1. NORMAL SUBJECTS

A. The hundred normal subjects used as a comparison group for the initial studies performed on large plates were those published by Clarke and Freeman (1968). Large plates were used in the studies on active chronic hepatitis, alcoholic cirrhosis, cryptogenic cirrhosis, the first series of patients with haemochromatosis and the first transplant patient. All other measurements were performed on miniplates.

B. The normal subjects for the miniplate studies consisted of healthy numbers of the laboratory staff (15) and blood donors (55) who were attending a Blood Transfusion Service donor session. Only subjects of Caucasian origin were accepted and care was taken to exclude women who were taking the contraceptive pill and those who had had an infection in the preceding three weeks. There were 37 males and 33 females their ages ranging from 20 to 69 years. The distribution of ages is shown in Table 5. Serum was separated within 6 hours of collection and stored without preservative at -20°C.

Table 5. Distribution of ages of normal subjects

Age	Males	Females	Total
20-29	15	11	26
30-39	11	5	16
40-49	5	5	10
50-59	5	7	12
60-70	1	5	6
			70

2. PATIENT GROUPS

A. Acute viral hepatitis

There were sera from 12 patients with typical viral hepatitis all of whom later made an uneventful recovery with return of liver function tests to normal. The diagnosis was confirmed by liver biopsy in 10 cases. Australia antigen was positive in 4 of the 10 cases tested. Details of the patients and their liver function tests at the time of the serum sample are shown in Table 6.

B. Active chronic hepatitis

There were sera from 20 patients with liver disease of greater than 3 months duration and biochemical evidence of disease activity (serum aspartate aminotransferase at least twice normal and serum gamma globulin >1.5g./100ml.) (Murray-Lyon et al, 1972). Liver biopsy material was obtained in 19 and all biopsies showed features of chronic aggressive hepatitis with established cirrhosis in 13 cases. In one patient biopsy was impossible because of persistent prolongation of the prothrombin time. Antibodies to mitochondria and smooth muscle were detected in 3 and 6 cases respectively and antinuclear factor was present in 13 cases. All cases were negative for Australia antigen. All patients were included in a prospective double blind trial comparing prednisone, 5mg. t.d.s. with azathioprine, 75mg. daily (Murray-Lyon, Stern and Williams, 1973). At the time the blood samples were taken 5 patients were receiving prednisone, 8 azathioprine and 8 were on no treatment immediately prior to inclusion in the trial. Details of the patients and liver function tests at the time of the serum sample are given in Table 7.

Serial samples from 4 further patients were taken before and at frequent intervals during treatment. Details of these cases are given in Appendix C.

Table 6. Clinical and biochemical details of the patients with viral hepatitis

Case	Sex	Age	Duration of jaundice (weeks)	LIVER FUNCTION TESTS* Bilirubin (mg/100ml)	Alkaline phosphatase (iu/l)	Aspartate transaminase (iu/l)	Total protein (g/100ml)	Albumin (g/100ml)	Prothrombin time (secs prolonged)
1	M	33	9	17	425	710	6.6	3.3	1
2	M	29	1	4.2	77	49	7.0	4.0	N.D.
3	F	22	1	9.6	95	180	6.9	3.8	N.D.
4	M	55	6	18	76	117	6.7	3.9	0
5	F	24	2	4.8	370	186	6.2	2.2	11
6	M	30	2	11	100	1150	5.4	2.6	4
7	M	41	2	10	77	2070	6.3	3.6	2
8	M	33	1	10	240	250	6.4	2.7	1
9	F	23	8	2.0	132	81	7.0	3.0	1
10	M	56	3	6.7	143	750	6.3	2.5	1
11	M	41	6	3.1	130	60	6.4	3.6	0
12	M	49	1	5.6	160	203	7.4	4.4	N.D.

N.D. = not done.

* Normal ranges serum alkaline phosphatase 30–85 iu/l.
serum aspartate transaminase 20–50 iu/l.

Table 7. Clinical and biochemical details of the patients with active chronic hepatitis

Case	Sex	Age	LIVER FUNCTION TESTS					
			Bilirubin (mg/100ml)	Alkaline phosphatase (iu/l)	Aspartate transaminase (iu/l)	Total protein (g/100ml)	Albumin (g/100ml)	Prothrombin time (secs prolonged)
1	F	50	0.5	55	136	8.9	3.26	2
2	F	50	10.5	61	91	8.8	3.0	1
3	F	29	0.5	55	60	8.6	3.6	2
4	F	20	0.5	61	52	8.0	3.3	0
5	M	34	20.3	172	382	7.3	3.2	3
6	F	57	0.5	61	169	9.3	3.3	1
7	F	75	0.5	73	86	8.4	3.2	1
8	M	19	0.5	68	63	7.9	2.9	1
9	F	67	1.5	92	370	7.4	2.9	3
10	F	14	0.5	135	85	8.0	3.2	1
11	M	29	2.2	184	136	10.0	3.4	0
12	F	58	3.2	73	221	10.5	5.1	1
13	F	48	0.5	172	133	8.4	3.1	1
14	F	30	1.2	178	191	9.5	4.0	1
15	F	56	1.1	86	37	8.0	3.6	3
16	F	47	1.0	55	113	7.6	3.3	1
17	M	14	3.4	172	843	12.0	3.1	3
18	F	42	5.9	98	128	8.1	2.8	1
19	M	20	2.5	104	133	6.6	2.7	4
20	F	73	1.5	155	210	6.7	1.9	3

Normal ranges: serum alkaline phosphatase 30–85 iu/l.
serum aspartate transaminase 20–50 iu/l.

C. Cryptogenic cirrhosis

There were sera from 12 patients with established cirrhosis for which there was no known cause (Murray-Lyon, et al, 1972). Liver biopsies in 11 showed little if any chronic inflammatory cell infiltrate (biopsy in one case was precluded by prolongation of the prothrombin time) and auto-antibodies were either negative or present in low titre. Clinical and biochemical details of these cases are shown in table 8.

D. Alcoholic Cirrhosis

Sera were examined from 12 patients with long histories of excessive alcohol intake all of whom had biopsy proven cirrhosis (Murray-Lyon, et al, 1972). Clinical details are given in Table 9.

E. Extrahepatic obstructive jaundice

Sera from 12 patients who later had an extrahepatic cause for obstructive jaundice demonstrated at laparotomy were studied. Clinical and biochemical details are shown in Table 10. The group of diseases represented is highly selected because of the interest of the Liver Unit.

F. Primary biliary cirrhosis

There were sera from 19 females and 1 male patient with primary biliary cirrhosis. Liver biopsy material in each case was compatible with the diagnosis (cirrhosis was established in 12 cases) and mito-chondrial antibodies were present in all patients. Nine have since died with hepatic failure and the autopsy findings confirmed primary biliary cirrhosis. Case 10 also had clinical features of scleroderma (Murray-Lyon, Thompson, Ansell and Williams, 1970). Details of the patients at the time of the serum sample are given in Table 11.

Table 8. Clinical and biochemical details of the patients with cryptogenic cirrhosis

			LIVER FUNCTION TESTS					
Case	Sex	Age	Bilirubin (mg/100ml)	Alkaline phosphatase (iu/l)	Aspartate transaminase (iu/l)	Total protein (g/100ml)	Albumin (g/100ml)	Prothrombin time (secs prolonged)
1	M	54	1.7	55	113	6.2	3.2	1
2	F	58	2.5	154	76	5.9	3.4	1
3	M	56	1.2	86	105	6.0	2.6	1
4	F	42	3.2	68	100	6.1	3.7	6
5	F	45	0.5	117	71	6.3	3.1	0
6	M	70	0.5	98	25	6.4	2.7	1
7	M	67	0.5	90	120	7.5	4.3	1
8	M	65	1.4	80	75	6.7	3.6	3
9	M	57	2.2	105	95	6.5	2.9	6
10	M	62	0.4	310	45	7.6	2.6	0
11	F	37	2.0	160	75	6.8	2.8	1
12	M	48	0.8	50	75	7.3	3.5	2

Normal ranges: serum alkaline phosphatase 30-85 iu/l
serum aspartate transaminase 20-50 iu/l

Table 9. Clinical and biochemical details of the patients with alcoholic cirrhosis

Case	Sex	Age	Bilirubin (mg/100ml)	Alkaline phosphatase (iu/l)	Aspartate transaminase (iu/l)	Total protein (g/100ml)	Albumin (g/100ml)	Prothrombin time (secs prolonged)
1	F	49	0.5	92	26	6.9	2.2	4
2	F	73	1.0	117	65	8.3	3.5	1
3	M	42	0.5	74	65	6.8	2.8	3
4	F	55	2.7	98	50	5.3	2.1	2
5	F	64	0.5	26	6	8.3	3.1	1
6	M	67	6.6	55	186	7.0	2.6	3
7	M	48	0.8	31	22	7.5	3.4	1
8	M	54	4.0	92	83	7.6	2.6	5
9	M	62	0.4	80	158	7.3	3.3	1
10	M	42	1.6	110	65	8.5	2.9	4
11	M	48	1.6	120	70	9.5	2.8	3
12	M	67	1.9	35	144	5.6	2.6	4

Normal ranges: serum alkaline phosphatase 30-85 iu/l.
serum aspartate transaminase 20-50 iu/l.

Table 10. Clinical and biochemical details of 12 patients with extrahepatic obstructive jaundice.

Case	Sex	Age	Cause of obstruction	Duration of jaundice (weeks)	LIVER FUNCTION TESTS: Bilirubin (mg/100ml)	Alkaline phosphatase (iu/l)	Aspartate transaminase (iu/l)	Total protein (g/100ml)	Albumin (g/100ml)	Cholesterol (mg/100ml)
1	M	66	Ca. hepatic ducts	24	12	1750	240	7.0	1.6	340
2	M	58	Ca. hepatic ducts	20	28	1900	200	6.6	3.2	600
3	M	42	Ca. hepatic ducts	8	21	625	90	5.6	2.9	550
4	F	55	Ca. hepatic ducts	12	19	1200	150	5.8	2.8	410
5	F	62	Ca. hepatic ducts	8	11	775	137	6.4	3.0	290
6	M	51	Ca. pancreas	8	16	245	110	6.2	2.3	330
7	F	44	Ca. hepatic ducts	8	24	154	80	6.8	2.8	330
8	F	78	Ca. hepatic ducts	8	23	497	147	5.8	1.8	820
9	M	52	Ca. hepatic ducts	8	15	434	70	6.8	1.6	290
10	M	58	Ca. pancreas	2	9.5	1170	120	6.8	3.0	520
11	M	80	Ca. C.B.D.	2	23	700	76	6.4	3.0	580
12	M	81	Calculi	1	6	49	56	6.5	4.0	N.D.

N.D. = not done

Table 11. Clinical and biochemical details of 20 patients with primary biliary cirrhosis

Case	Age	Sex	Duration of illness (months)	LIVER FUNCTION TESTS						
				Bilirubin (mg/100ml)	Alkaline phosphatase (iu/l)	Aspartate transaminase (iu/l)	Total protein (g/100ml)	Albumin (g/100ml)	Prothrombin time (secs prolonged)	Cholesterol (mg/100ml)
1	50	F	60	25.0	310	650	6.0	1.2	7	200
2	48	F	60	11.0	340	220	7.2	2.8	3	350
3	58	F	36	1.7	189	30	6.4	3.4	0	320
4	38	F	24	4.2	800	150	8.6	3.3	1	700
5	29	F	72	1.2	800	174	7.1	2.9	0	267
6	63	F	18	4.9	800	310	8.1	3.1	1	300
7	50	F	120	6.9	500	220	8.0	3.6	3	280
8	63	M	48	19.0	775	410	8.5	2.1	2	250
9	60	F	48	0.7	625	125	7.5	4.1	0	180
10	70	F	48	4.8	875	100	7.8	2.8	1	360
11	63	F	24	0.4	675	99	8.1	3.5	2	267
12	51	F	18	50.0	2000	1500	6.7	3.2	3	700
13	47	F	36	19.0	375	370	5.8	2.5	6	250
14	53	F	12	1.3	380	105	7.6	2.7	2	250
15	43	F	12	1.0	860	260	8.5	4.0	1	400
16	64	F	24	00.6	350	70	7.3	3.6	1	220
17	61	F	48	23.0	475	210	6.4	1.5	10	500
18	57	F	6	6.4	338	76	6.3	2.8	5	252
19	28	F	18	0.6	350	172	8.0	3.6	0	407
20	70	F	84	3.7	650	220	7.1	3.0	2	340

G. Primary haemochromatosis

i) Sera from a series of 29 patients were studied on large plates (Amin, Clarke, Freeman, Murray-Lyon, Smith and Williams, 1970). Details of the cases are given in Table 12. Except for case 10 all patients were male. Nine had clinical or radiological evidence of arthropathy.

ii) A further series of 40 sera were examined on miniplates. These included 12 subsequent specimens taken about 3 years later from patients in the first series (Table 13). All cases except no. 38 were male. Twelve of the 28 new patients had clinical or radiological evidence of arthropathy. In cases 30-49 thirteen individual proteins were measured in each case but in cases 50-69 only serum haemopexin was quantitated.

Thus a total of 69 sera from 57 patients were examined. The diagnosis was confirmed in each case by liver biopsy which showed grade IV iron overload with extensive fibrosis (Scheuer, Williams and Muir, 1962). Established cirrhosis with nodular regeneration was demonstrated unequivocally in 38 of the 57 patients. Liver function tests were within normal limits in the great majority of cases.

iii) Serial blood samples were obtained from cases 30 and 33-37 before and at approximately monthly intervals during venesection.

H. Relatives of patients with primary haemochromatosis

The sera from 14 first and second degree relatives were examined. Cases 9, 12-14 had had a few pints of blood venesected 3 or more years previously. None had clinical or biochemical evidence of liver disease but the serum iron was raised in some cases (Table 14).

I. Secondary iron overload

Sera from 15 patients with secondary iron overload from a variety of causes were examined and clinical details of the cases are given in Table 15.

Table 12. Clinical and laboratory details of 29 patients in first series of patients with primary haemochromatosis

Case	Age	Duration of illness (years)	Diabetes mellitus	Serum iron (μg/100ml)	Total iron binding capacity (μg/100ml)	DFO* (μg/Kg)	Last venesection (years)	Cirrhosis
1	58	6	+	40	275	173	current	±
2	38	7	+	235	260	1172	1/12	+
3	70	16	+	225	255	2021	current	+
4	50	14	0	225	260	1027	3	+
5	39	5	0	205	215	1039	3	+
6	69	14	+	280	345	838	6/12	+
7	64	12	0	255	265	825	6	+
8	48	2	+	50	330	523	current	+
9	70	8	+	140	230	512	6	+
10	74	2	+	100	255	455	1	+
11	73	7	+	172	315	249	6	0
12	62	8	+	145	210	537	6	+
13	48	2	0	190	205	1539	1/12	+
14	54	5	+	290	300	744	1/12	0
15	74	10	0	230	240	837	8	0
16	49	11	0	280	360	268	1	0
17	46	6	+	160	190	3029	0	+
18	38	3	+	235	240	1946	current	+
19	72	4	+	95	315	310	current	+
20	62	11	+	70	330	81	9/12	+
21	39	3	0	25	450	110	1/12	+
22	65	3	+	210	240	1444	5/12	±
23	57	1	+	215	235	1372	current	±
24	54	7	+	145	285	120	4/12	+
25	69	15	0	310	350	463	13	±
26	49	2	+	180	285	791	current	+
27	60	10	+	95	310	271	current	0
28	49	4	0	110	225	371	current	+
29	55	8	0	155	400	302	1	0

* DFO = differential ferioxamine test. Normal <300 μg/Kg

Table 13. Clinical and laboratory details of the second series of patients with primary haemochromatosis

Case No.	(Previous No.)	Age	Duration of illness (years)	Diabetes mellitus	Serum iron (µg/100ml)	Total iron binding capacity (µg/100ml)	DFO* (µg/Kg)	Last vene-section (years)	Cirrhosis
30		61	8	+	270	320	1633	4	+
31		54	4	0	250	300	1700	1	+
32		46	4	0	230	230	1692	0	0
33		62	2	0	170	180	>862	0	+
34		55	11	+	210	235	961	4	+
35		56	14	+	270	300	3303	0	±
36		71	1	0	380	380	719	0	0
37	(27)	64	14	+	190	220	1196	3.5	0
38		84	4	0	260	280	1764	0	+
39		34	1	0	40	340	94	1/52	+
40		47	6	+	230	265	796	3.5	+
41		57	4	+	215	260	2040	0	+
42		52	16	+	290	315	1492	5	+
43	(19)	76	8	+	255	345	1212	4	+
44		37	2.5	+	260	325	636	current	+
45	(21)	43	7	0	250	275	809	3.5	+
46	(25)	73	19	0	270	310	1890	4	±
47	(24)	57	10	+	210	235	66	3	+
48		43	10	0	180	200	1500	0	+
49	(18)	41	6	+	-	-	284	2	+
50		69	1	+	115	140	1012	0	+
51		68	18	0	215	300	947	3	+
52		49	12	+	220	250	418	3.5	0
53		40	4	0	255	280	762	3	0
54		40	1	0	-	-	1154	current	0
55		61	5	0	50	240	419	3	0
56		48	3.5	+	280	300	847	1.5	+
57	(24)	57	10	+	210	235	66	3	+
58		62	2	0	200	220	1072	current	+
59		51	7	+	245	350	797	3	+
60		38	3	0	290	295	796	9/12	+
61		67	19	0	120	175	902	2	0
62		78	8	+	160	185	1457	4	+
63		60	11	+	245	325	1102	2	±
64	(23)	61	5	+	235	270	1347	2	±
65	(28)	53	7	0	225	270	750	4	+
66	(22)	69	7	+	95	220	838	3	+
67	(26)	53	6	+	245	250	1208	2.5	+
68	(20)	65	14	+	215	245	1025	4.5	+
69		60	1/12	0	165	235	3131	0	+

* DFO = differential ferrioxamine test.

Table 14. Clinical and laboratory details on relatives of patients with primary haemochromatosis

No.	Age	Sex	Relationship to propositus	Serum iron (µg/100ml)	Total iron binding capacity (µg/100ml)	Differential ferrioxamine test (µg/Kg)
1	50	M	Sibling	180	350	338
2	44	M	Nephew	145	325	431
3	42	M	Son	105	315	208
4	73	M	Sibling	195	295	263
5	41	M	Nephew	100	265	151
6	18	M	Son	95	310	-
7	55	M	Nephew	155	400	302
8	59	M	Son	145	295	266
9	54	M	Son	140	250	117
10	65	M	Father	70	350	-
11	60	F	Mother	85	370	-
12	65	M	Sibling	185	310	303
13	71	M	Sibling	205	270	530
14	67	M	Sibling	115	355	475

Table 15. Clinical and laboratory details on patients with secondary iron overload

No.	Age	Sex	Diagnosis	Serum iron (µg/100ml)	Total iron binding capacity (µg/100ml)	Differential ferrioxamine test (µg/Kg)	Comment
1	48	M	Alcoholic cirrhosis	105	270	1037	Liver biopsy grade 2 iron
2	45	M	Unknown	180	205	1012	Liver biopsy grade 3 iron + fibrosis
3	35	M	Alcoholic cirrhosis	60	310	513	Liver biopsy grade 2 iron
4	58	F	Cryptogenic cirrhosis + PCT	70	370	218	Being venesected
5	66	M	Alcoholic cirrhosis	105	250	434	Previously bled
6	60	M	Unknown	195	235	867	Liver biopsy grade 3 iron
7	66	M	Alcoholic PCT	100	360	293	Previously bled Liver fibrosis
8	31	M	Unknown	220	250	219	Iron stores unaltered after 4 years
9	60	F	Alcoholic PCT	180	400	382	Previously bled
10	55	F	Excess oral iron	160	230	1013	Liver biopsy cirrhosis. Grade 3 iron
11	57	M	Chronic active hepatitis	225	340	942	Liver biopsy Grade 2 iron
12	57	M	Aplastic anaemia	220	260	-	Multiple transfusions (> 20 units)
13	12	F	Aplastic anaemia	210	397	-	Transfused 21 units blood
14	83	F	Myelofibrosis	140	195	-	Transfused 29 units blood
15	24	F	Myelofibrosis	98	241	-	Transfused 30 units blood

PCT = porphyria cutanea tarda

Cases 2 and 6 were not considered to have primary haemochromatosis as there were no abnormal physical signs and the degree of hepatic fibrosis was slight.

J. Controls and patients with autoimmune liver disease studied for evidence of in vivo activation of complement

Three healthy laboratory personnel and 3 patients in the primary biliary cirrhosis series (patients no. 5, 9, 18) were studied. In addition there were 6 patients with biopsy proven active chronic hepatitis including Cases 1, 3 and 4 (Appendix C) and Cases 5 and 18 (Table 7). Three of these cases were positive for Australia antigen and the other 3 cases had autoantibodies to smooth muscle, mitochondria or antinuclear factor.

K. Liver transplant recipients

Serum specimens were studied before and at intervals following orthotopic liver transplanation in 4 recipients. Detailed case histories are found in Appendix D. Case 1 was studied using large plates (Murray-Lyon, Clarke, Freeman and Williams, 1970) and cases 2-4 using miniplates.

L. Patients with severe deficiency of plasma α_1-antitrypsin

Serum samples from 7 adult subjects, 4 of whom had severe emphysema were studied. Two were asymptomatic siblings (Table 16) and one elderly man had slight bronchitis but no evidence of emphysema on detailed pulmonary assessment (Hutchison, et al, 1971). None had overt pulmonary infection at the time samples were taken and none had clinical evidence of liver disease. No patient had a family history of liver disease.

M. Effects of enzyme inducing drugs

i) Normal subjects

Serial blood samples were taken from 2 normal subjects before and at

Table 16. Clinical and laboratory details of adult patients with alpha-1-antitrypsin deficiency

Case No.	(BMJ* Case No.)	Age	Sex	Clinical state	Liver function tests
1	(7)	42	M	Severe emphysema	Bilirubin 1.8mg/100ml.
2	(5)	52	M	Severe emphysema	Normal
3	(5b)	47	F	Normal. Sib of Case 2	Normal
4	(4)	48	M	Severe emphysema	Normal
5	(4b)	53	M	Normal. Sib of Case 4	Normal
6	(3b)	72	M	Slight bronchitis	Normal
7	(6)	54	F	Severe emphysema	Normal

* Nos. referring to patients in article by Hutchison et al (1971)

intervals following courses of Bucolome (Butymidin; Takeda Chemical Industries, Ltd., Osaka, Japan) and N-phenylphetharbital (Phetharbital) drugs which are said to induce a variety of hepatic enzyme systems. The development of enzyme induction was assessed by serial measurement of plasma bilirubin (Hunter, et al, 1971c) and urinary D-glucaric acid excretion (Hunter, et al, 1971a).

ii) Epileptics on anticonvulsant therapy

Fifteen patients with severe epilepsy all of whom had been treated with anticonvulsant therapy for longer than a year were also studied. In addition to serum samples for immunoelectrophoresis and routine biochemical screening, urine was obtained for estimation of D-glucaric acid excretion. Details of the patients are given in Table 17. To obtain a measure of the total daily dose of anticonvulsant, a simple scoring system was used, one unit being ascribed to every 30mg phenobarbitone, 50mg. diphenylhydantoin, 100mg. primidone, 250mg. ethosuximide.

Table 17. Clinical and laboratory details of 15 adult epileptic patients on long term anticonvulsant therapy.

Case No.	Age	Sex	No. of different anticonvulsant drugs	Amount of anticonvulsant units	Alkaline* phosphatase (iu/l)	D-glucaric acid output µg/g + creatinine
1	17	M	2	3	128	10.5
2	45	M	3	11.5	104	16.3
3	37	F	2	9	54	55.9
4	37	F	3	8	72	12.4
5	51	M	2	18.5	76	119.0
6	50	M	2	9	100	25.5
7	17	M	2	9	155	26.0
8	18	F	3	13	92	15.8
9	25	M	2	11.5	56	16.0
10	48	M	2	8.0	84	28.0
11	26	F	2	13.5	67	21.5
12	35	M	2	13.5	124	69.0
13	38	M	2	15.5	66	23.8
14	22	M	2	15.5	69	-
15	60	M	3	4	88	8.3

* normal range:- 30-85 iu/l + 6-10 µg/g creatinine

3. STATISTICAL METHODS

In the early studies using large plates clinical and laboratory data was collected and categorised according to the protocol in Appendix E. The values for all proteins except haptoglobin and haemopexin were log transformed as the distribution of the protein levels was log-normal (Healey, 1968). In the first study on patients with primary haemochromatosis the protein levels for each patient were analyzed statistically for correlations amongst proteins and between protein changes and all the clinical and laboratory data recorded. In the subsequent large plate studies the number of clinical and laboratory indicants used in the correlation matrix was limited to those in Table 18.

Table 18

Clinical and laboratory data analysed for correlations with the concentrations of each plasma protein in the large plate studies

Age
Duration of illness
Current treatment with corticosteroids
Skin stigmata of chronic parenchymal disease
Presence of hepatic encephalopathy
Presence of ascites
Erythrocyte sedimentation rate (E.S.R.)
Prothrombin time
Serum bilirubin
Serum alkaline phosphatase
Serum aspartate aminotransferase
Serum albumin
Total plasma protein
Presence or absence of cirrhosis on liver biopsy
Presence of autoantibodies

In the miniplate studies all protein values were log transformed and the clinical and laboratory data analysed were restricted to the age and sex of the patient, duration of history, liver function tests, prothrombin time, albumin and total protein. Other data where relevant to special groups of patients are shown in the tables.

Standard statistical techniques were used throughout with the aid of the London University Computer and the Glasgow University Computer.

The diagnostic value of the protein values was assessed by the error in reclassification of the patients using a stepwise discriminant analysis (Dixon, 1968; Programme BMD07M) and a Bayesian model (Knill-Jones, R.P.; personal communication). The error in reclassification was compared with the results using only data from the cellulose acetate electrophoresis strips.

PART 5

RESULTS

RESULTS

1. Protein concentrations in the control population and normal subjects

A. Normal range

The mean and standard deviation (S.D.) of the concentrations of the 13 proteins measured in the 70 control subjects by the miniplate technique are given in Table 19. The values for β lipoprotein are expressed as a percentage of the acetylated albumin marker as β lipoprotein does not appear in the standard serum. All other proteins are given as a percentage of the concentration of that protein in the standard serum.

3 subjects had α_1-antitrypsin concentrations outwith the normal range (63-133%) established on the group as a whole. Their serum was examined by starch gel electrophoresis (Dr. P.J.L. Cook) and all 3 were heterozygous deficient, their Pi types being MZ (2) and SZ (1).

The coefficient of variation for most proteins lay between 10-20% with a greater variability for haptoglobin and caeruloplasmin.

The absolute value of the mean and normal range of 5 proteins measured in the control population was determined by comparison with the results of immunoelectrophoresis of the Hoechst standard serum for which absolute concentrations of certain proteins are provided. For each protein there was a close agreement (Table 20) with the normal values obtained by a variety of immunological methods given in the literature.

B. Variation in protein concentration with sex

The mean and standard deviation of the 13 proteins (expressed as per cent acetylated albumin) were analysed for males and females separately and significant differences were found for only 3.

Table 19. Normal range for 13 proteins measured in 70 control subjects and expressed as % standard serum.

Protein	Mean	S.D.	Normal range Mean ± 2 S.D.
Prealb.	111.1	23.2	64.7 - 157.5
PGP	103.0	15.5	72.0 - 134.0
α_1AT	98.9	17.2	63.5 - 133.3
GC	101.0	15.7	69.6 - 132.4
α_2M	120.6	28.7	63.2 - 178.0
Caer.	133.8	34.8	64.2 - 203.4
10	109.3	20.7	67.9 - 150.7
Hpt	65.1	27.4	10.3 - 119.9
Hpx	107.2	18.4	70.4 - 144.0
Trf	108.5	17.4	74.7 - 143.3
*βLP	21.7	5.3	11.1 - 42.3
16	103.3	18.4	66.5 - 140.1
18	108.9	13.8	81.3 - 136.5

* expressed as % acetylated albumin marker

Table 20. Absolute values (mg/100ml) for 6 proteins in the 70 normal subjects compared with normal values in the literature.

Protein	Present study Mean (mg/100ml)	S.D.	Other series Mean (mg/100ml)	S.D.	Author
Prealb.	26	5.5	25	7.5	Storiko (1968)
			25	6	Rossi et al (1970)
			25	6.5	Weeke and Knasilnikoff (1972)
			22	3	Ingenbleek et al (1972)
GC	23.4	3.5	39.8	-	Cleve and Dencker (1967)
			35	5	Storiko (1968)
			25	-	Hughes (1971)
			27	5	Weeke and Knasilnikoff (1972)
α_2^M	186	44	180	-	Ganrot and Schersten (1967)
			200	24	Adham et al (1968)
			240	55	Storiko (1968)
			284	89	Housley (1968)
Hpt	87	36	160 (range 10-220)		Storiko (1968)
			- (range 55-357)		Lyngbye and Kroll (1971)
			147 (range 58-373)		Weeke and Knasilnikoff (1972)
Hpx	81	12.8	- (range 66-100)		Hanstein and Muller-Eberhard (1968)
			100 (range 80-130)		Storiko (1968)
			81 (range 59-103)		Braun and Aly (1971)
			80 (range 53-121		Weeke and Knasilnikoff (1972)
Trf	242	38	270	45	Fiaschi et al (1969)
			- (range 179-335)		Lyngbye and Kroll (1971)
			226 (range 152-336)		Weeke and Knasilnikoff (1972)

Table 21. Proteins with significant difference between mean concentrations in males and females

Protein	Males Mean	Males S.D.	Females Mean	Females S.D.	Significance
Prealb.	208.5	7.0	198.5	8.0	$p < 0.002$
α_2M	204.4	9.6	210.2	8.8	$p < 0.01$
Caer	207.5	8.5	215.7	10.9	$p < 0.002$

C. Variation with age

A plot of the concentration of each protein against age showed a distinct trend for easily precipitable glycoprotein and haemopexin to rise with age and there was a significant difference between the mean values in the age groups 20-44 and 45-70 for both these proteins ($p < 0.002$). The concentration of the other proteins did not appear to vary with age.

D. Correlations between individual proteins in normal sera

Significant correlations ($r > 0.5$; $p < 0.001$) were found between the following pairs of proteins in the population of 70 normal subjects

GC:Prealb.	Hpx:18	Trf:Hpx
PGP:16	Hpx:16	Trf:18
PGP:18	Hpx:Caer.	10:18
PGP:Caer.	Hpx:10	16:18
PGP:10	Trf:10	
PGP:Hpx	Trf:Prealb.	
PGP:Trf		

E. Variation in normal sera with repeated sampling

Repeated samples were taken from 5 members of the laboratory staff over a period of 9 months. The values for each subject varied little and remained within 2 S.D. of the mean established in 70 normals. In one subject in whom 10 samples were obtained the variance of the readings for most proteins was greater than the variance of repeated measurements of the same protein in the standard serum. However, the difference was

statistically significant only for group component and caeruloplasmin (Table 22).

F. Comment

The values for the 6 proteins expressed as mg/100ml serum agree well with the ranges determined by immunological methods for normal subjects given in the literature. Variation in the means in the different series is to some extent a reflection of the purity of the standard protein used to calibrate the system, for the use of an impure standard will give a spuriously high reading for the protein in the serum being tested. The 10-20% coefficient of variation for most of the 13 proteins measured also agrees well with the figure given by Clarke and Freeman (1968) and Ganrot (1972). The range for haptoglobin and therefore its coefficient of variation is well known to be large. The reason for this is not clear but it may be due to variable polymerisation of the protein.

Differences in plasma protein values according to age and sex have been studied in large numbers of subjects by Lyngbye and Kroll (1971) and Weeke and Krasilnikoff (1971). The concentration of some proteins is dependent on both age and sex and complex polynomial regressions have been derived to express this variation (Weeke and Krasilnikoff 1971). Protein concentrations in infants (Abrams and Freeman, 1969) and children (Abrams, 1971) differ greatly from adults. Considering only adults the rise in haemopexin with age in the present series is in agreement with Weeke and Krasilnikoff (1971) although Clarke and Freeman (1968) found no change with age, but as in this study both groups found a rise in easily precipitable glycoprotein. Most authors agree that prealbumin is slightly higher in adult males than females and that α_2-macroglobulin and caeruloplasmin are higher in the female (Rossi et al, 1968; Stabilini et al, 1968; Clarke and Freeman, 1968; Weeke and Krasilnikoff, 1971, Ganrot, 1972).

Table 22. Variation in protein concentrations (expressed as per cent acetylated albumin) in repeated samples taken from a normal subject compared with repeated estimations of the standard serum.

	Normal subject			Standard serum	
Protein	Mean (n=10)	S.D.	Significance	Mean (n=12)	S.D.
PGP	36.8	4.64		39.9	5.5
α_1AT	115.4	10.17		122.5	8.6
GC	125.3	19.2	**	124.2	7.7
α_2M	24.8	3.26		22.6	2.0
Caer.	67.9	9.77	*	57.7	4.5
10	25.1	3.93		23.5	4.5
Hpt	137.6	44.68		199.0	30.0
Hpx	36.8	4.18		37.1	3.7
Trf	38.5	5.3		38.8	3.9
16	54.3	5.68		47.6	4.8
18	54.2	5.43		49.4	4.8

* $F = 4.8; p \leq 0.05$ ** $F=6.4; p \leq 0.01$

The present results are in agreement with these trends. However any differences in the mean values between the sexes are not great and Lyngbye and Kroll (1971) and Adham, et al (1971) found no differences for prealbumin and $\alpha_2$macroglobulin respectively. Other factors which may influence the concentration of some plasma proteins are whether or not the subject is fasting, the time of day and the season of the year (Lyngbye and Kroll 1971). It was not practical to take account of these factors in the present study.

As the differences in the protein concentrations were shown to vary little with age or sex the control group as a whole has been used for comparison with the patient groups.

Various workers have published correlation matrices for the different plasma proteins in normal subjects (Phillips and Blackmore, 1971; Ganrot, 1972; Werner et al, 1972). Clearly because of the large number of correlations examined a certain number will achieve conventional statistical significance by chance. Therefore only those arising by chance less than once in a thousand times are given. No ready biological explanation is apparent for the findings and the pattern of correlations is different in each of the 4 matrices examined.

2. PROTEIN CONCENTRATION IN DIFFERENT PATHOLOGICAL STATES

A. Relation to necrosis and inflammation

i) Acute viral hepatitis.

There were significantly increased concentrations of easily precipitable glycoprotein and $\alpha_1$antitrypsin, caeruloplasmin, proteins 10 and β lipoprotein. The levels of prealbumin, haptoglobin and haemopexin were significantly reduced (Table 23).

Correlations were sought between the protein levels and the length of history and liver function tests. There were significant negative

Table 23. Mean protein levels (± S.D.) expressed as per cent standard serum in acute viral hepatitis (miniplates) and active chronic hepatitis (large plates)

Protein	Viral Hepatitis (n=12)	Normal subjects (n=70)	Active chronic hepatitis (n=20)	Normal subjects (n=100)
Prealb.	56 (40)***	111 (23)	-	-
PGP	182 (44)***	102 (15)	151 (42)***	115 (19)
α_1AT	136 (20)**	99 (17)	-	-
GC	91 (19)	101 (15)	105 (35)	117 (15)
α_2M	135 (41)	121 (29)	175 (51)***	122 (25)
Caer.	179 (67)*	133 (34)	175 (45)***	137 (25)
10	170 (55)***	109 (20)	143 (57)	121 (27)
Hpt	56 (46)*	65 (27)	48 (52)**	103 (44)
Hpx	58 (38)***	107 (18)	108 (38)	107 (13)
Trf	98 (22)	108 (17)	135 (39)**	110 (15)
βLP	†34 (11)*	†21 (5)	175 (67)	177 (43)
16	131 (32)	103 (18)	-	-
18	112 (33)	108 (13)	-	-
β_1AC	-	-	185 (87)**	107 (24)
Alb. (g/100ml)	3.46 (0.46)	-	3.2 (0.66)	-

† Expressed as % acetylated albumin

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

correlations ($p < 0.01$) between haemopexin and the serum aspartate aminotransferase and also the degree of prolongation of prothrombin time, and between protein 16 and the serum aspartate aminotransferase. There were significant positive correlations ($p < 0.01$) between prealbumin and haemopexin, prealbumin and protein 16, protein 10 and β lipoprotein, proteins 10 and 18, haptoglobin and protein 16.

ii) Active chronic hepatitis

a) Single samples in 20 patients. Six of the 10 proteins measured were significantly different from the values in the control population. The concentrations of easily precipitable glycoprotein, α_2-macroglobulin, caeruloplasmin, transferrin and β_1A globulin were increased and haptoglobin was decreased. An analysis of the protein levels with 15 indicants from the clinical and laboratory data (Table 18) showed a significant correlation only between both easily precipitable glycoprotein and transferrin and the alkaline phosphatase ($p < 0.001$).

b) Serial readings in four patients. The effects of treatment with azathioprine or corticosteroids were studied in 4 patients. Samples were taken before and for some moths after therapy was started. Details of the individual cases are recorded in Appendix C and the protein concentrations before treatment are given in Table 24.

Case 1. Before treatment 5 proteins were present at concentrations above normal (easily precipitable glycoprotein, α_1 antitrypsin, α_2-macroglobulin, caeruloplasmin and protein 10) and haemopexin and prealbumin concentrations were reduced (Table 24). Treatment with large doses of corticosteroids resulted in a fall in serum bilirubin to a minimum of 2.2mg/100ml after 5 months and accompanying this improvement there was a gradual rise in prealbumin and haemopexin concentrations into the normal range and a fall in the concentrations of caeruloplasmin, α_2-macroglobulin, and protein 10 to just within the normal range (Fig. 19). The concentrations

Table 24. Protein concentrations expressed as % standard serum in four patients with active chronic hepatitis before therapy.

Protein	Case Number 1	2	3	4	Normal range mean ± 2 S.D. (n=70)
Prealb.	32	20	50	-	65 - 158
α_1PGP	175	108	128	63	72 - 134
α_1AT	170	154	140	108	64 - 133
GC	103	36	95	34	70 - 132
α_2M	205	155	177	125	63 - 178
Caer	220	163	156	100	64 - 203
10	154	175	158	129	68 - 153
Hpt	61	18	3	0	10 - 120
Hpx	57	21	41	27	70 - 144
Trf	137	68	76	56	75 - 143
*βLP	40	28	35	13	11 - 42
16	135	73	54	86	67 - 140
18	98	16	64	30	81 - 137

* expressed as percentage of area of acetylated albumin

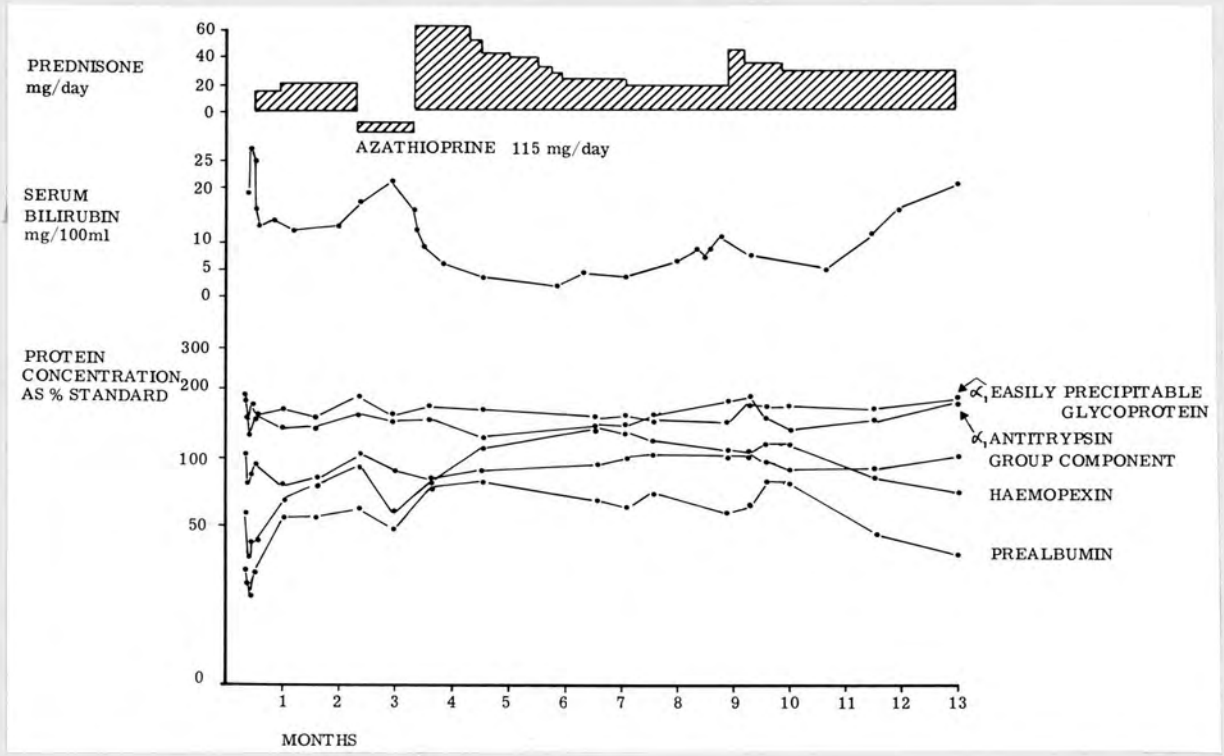


Figure 19. Case 1. Concentration of five proteins at intervals during treatment for active chronic hepatitis.

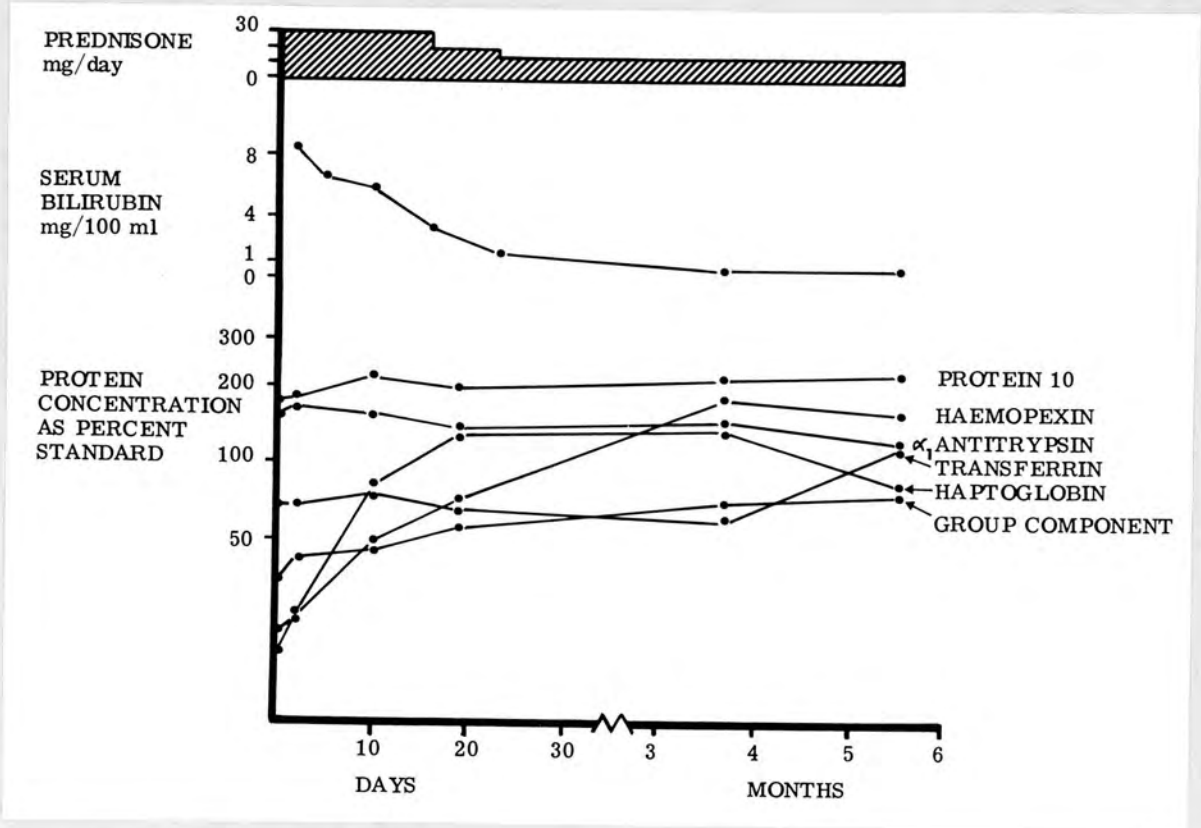


Figure 20. Case 2. Concentration of six proteins at intervals during treatment with corticosteroids.

of easily precipitable glycoprotein and α_1-antitrypsin remained high. During the subsequent deterioration in liver function the plasma protein pattern again became abnormal. The other proteins measured remained in the normal range throughout the period of study.

Case 2. Corticosteroid therapy resulted in gradual improvement in liver function tests and the concentration of 4 or 5 proteins which had been reduced (prealbumin, group component, haemopexin, protein 18 and transferrin) (Table 24) rose to normal or above normal concentration (Fig. 20). The concentration of haptoglobin also rose markedly. The levels of α_1-antitrypsin which had been increased returned to normal, but protein 10 remained high. At the end of 5 months treatment liver function was normal apart from slight elevation of aspartate aminotransferase (82 I.U./l.), and all proteins measured were within the normal range except for elevation of protein 10 and haemopexin.

Case 3. During treatment with azathioprine there was a slow fall in serum bilirubin and the raised levels of protein 10 returned to the normal range after 3 months but α_1-antitrypsin remained elevated (Fig.21). Prealbumin, haptoglobin and haemopexin remained low but proteins 16 and 18 rose to normal concentrations after 3 months. Other proteins remained within the normal range before and during treatment.

Case 4. Concentrations of easily precipitable glycoprotein, group component, haemopexin, transferrin and protein 18 were low and haptoglobin absent before treatment and there was little change over the next 6 weeks despite a progressive fall in serum bilirubin (Fig. 22). By 3 months when the serum bilirubin had fallen to 1.2 mg/100ml there was a striking rise in the concentrations of most proteins and haptoglobin was present for the first time. Levels of easily precipitable glycoprotein, $\alpha_2$macroglobulin and transferrin were high and only protein 18 was low. At 7 months the

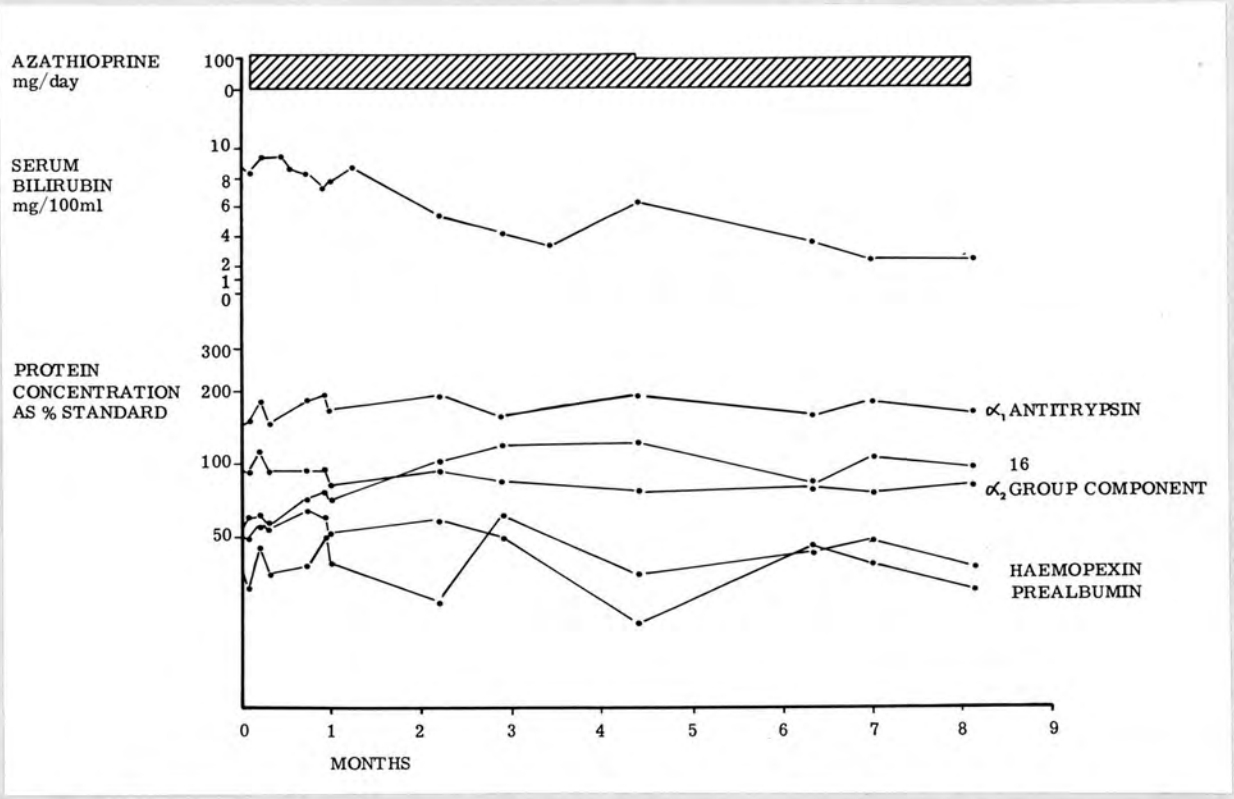


Figure 21. Case 3. Concentration of five proteins at intervals during treatment with azathioprine.

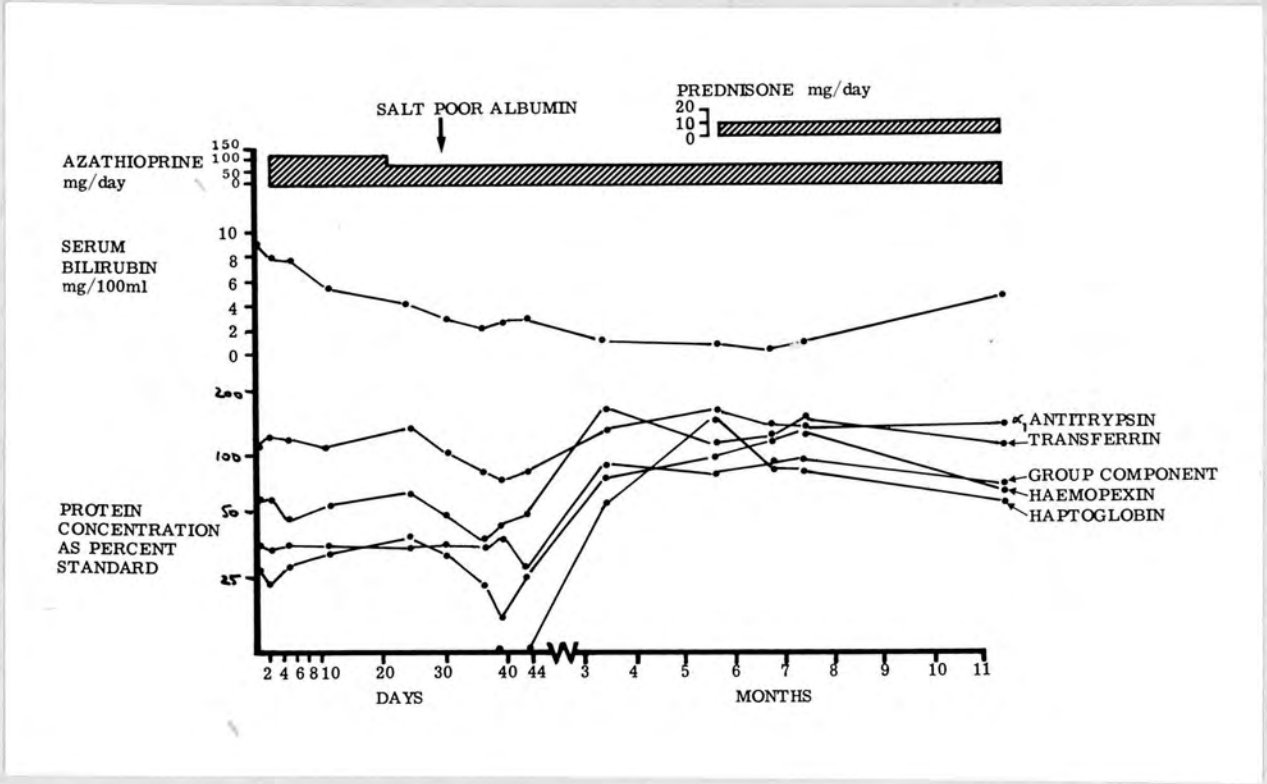


Figure 22. Case 4. Concentration of five proteins at intervals during treatment for active chronic hepatitis.

protein pattern was normal apart from raised levels of proteins 10 and 16. At 11 months the bilirubin had risen to 4.8mg/100ml and the protein pattern had become more abnormal with high levels of α_2-macroglobulin and protein 10 and low levels of group component, haemopexin and protein 18.

In all 14 serum samples examined the configuration of the prealbumin arc was abnormal with 2 or 3 subsidiary peaks trailing across the plate to the origin (Fig. 23). The shape was variable (Fig. 24) but never returned to normal and the degree of abnormality appeared to be related to the severity of disturbance in liver function. The latest sample taken when liver function had again deteriorated was similar to Fig. 23. Autoradiography was performed (Dr. H.M. Clarke) on a plate in which the serum applied was mixed with 0,2μCi ^{125}I thyroxine and this showed the protein bound thyroxine throughout the length of the arc (Fig. 25).

Samples of serum from the patient's 2 children showed a normal pattern with no disturbance of the prealbumin arc.

B. Relation to hepatic fibrosis

i) Inactive cryptogenic cirrhosis

Here the only significant difference in the 11 proteins measured from the normal values was a rise in α_2-macroglobulin and β_1AC globulin (Table 25).

ii) Alcoholic cirrhosis

There was a significant elevation of α_2-macroglobulin , β_1AC globulin and caeruloplasmin concentrations and transferrin was depressed below normal. (Table 25).

In neither group were there any significant correlations between the concentrations of any protein and the clinical and laboratory data in Table 18.

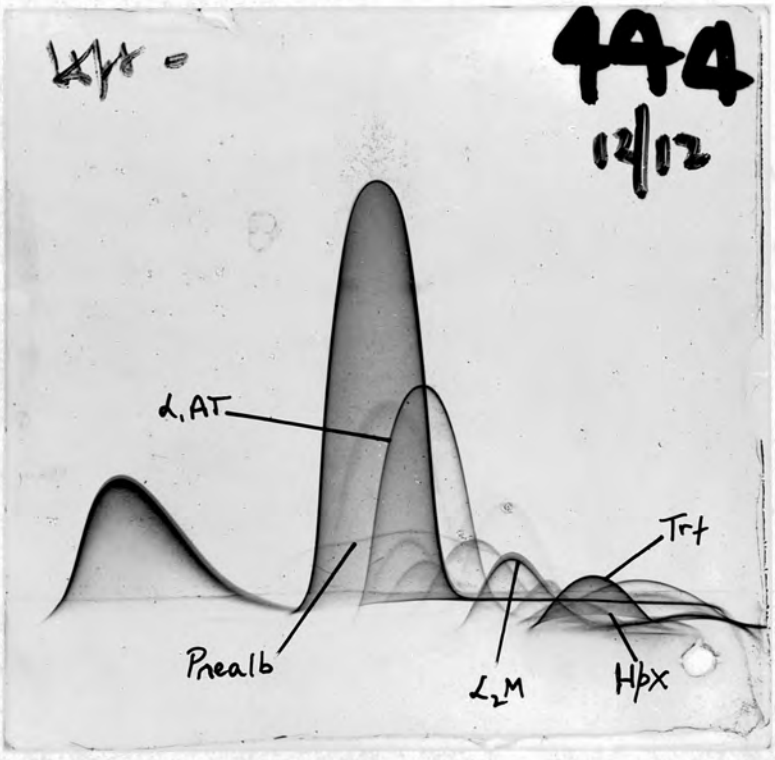


Figure 23. Case 4. Serum sample before treatment. Note the configuration of the prealbumin arc and absence of haptoglobin.

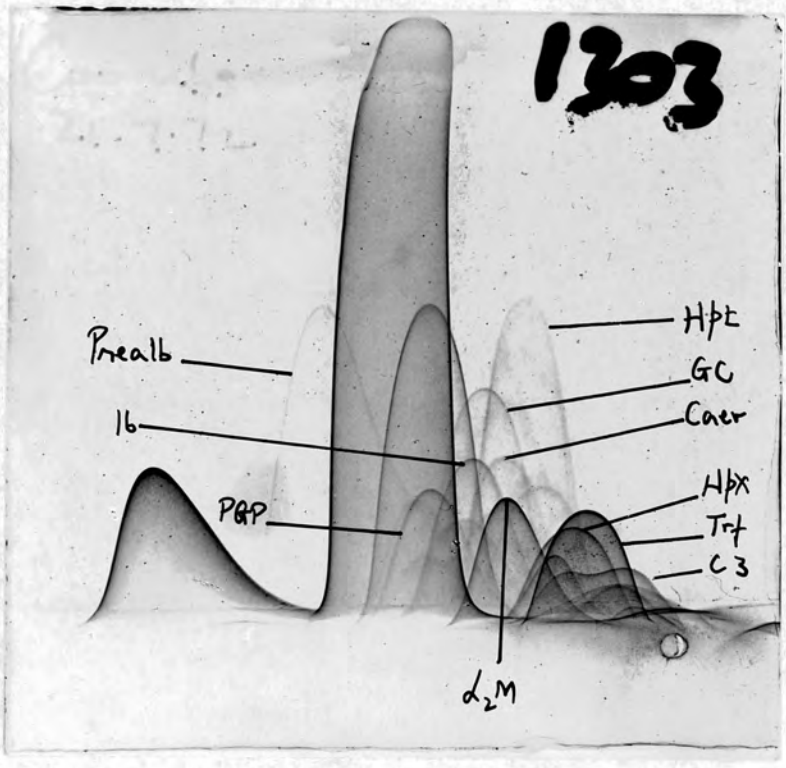


Figure 24. Case 4. Serum sample after seven months treatment. Most proteins are present at higher concentration and prealbumin arc is more normal.

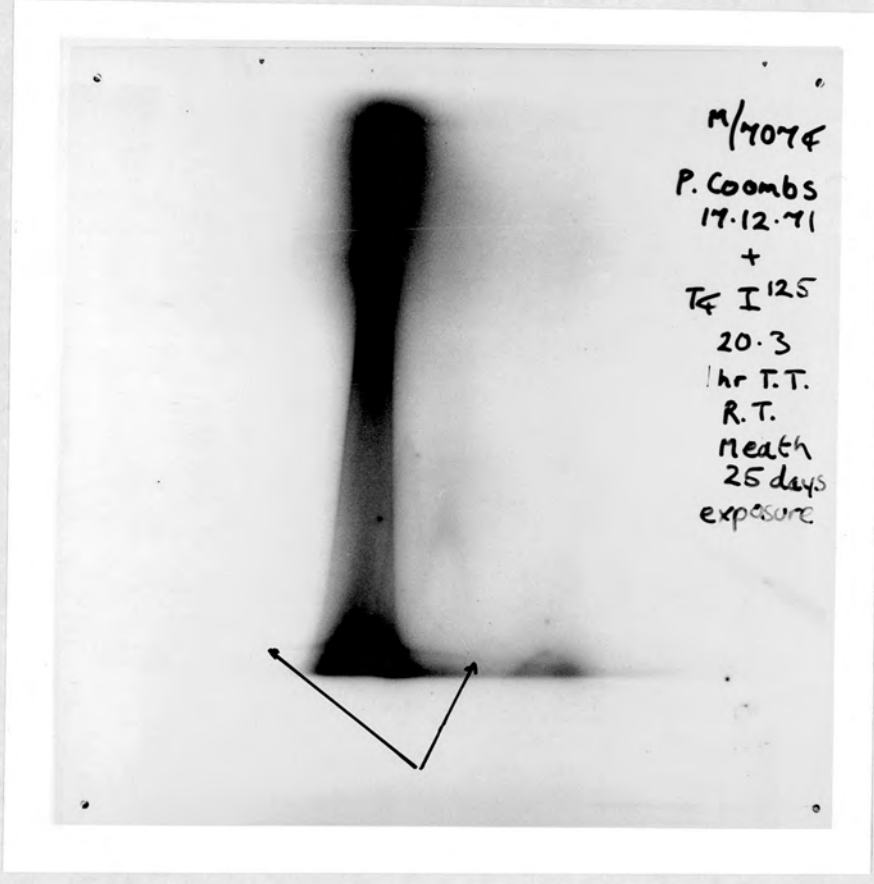


Figure 25. Case 4. Autoradiograph of plate with added radioactive thyroxine to show binding to the whole length of the abnormal prealbumin arc.

Table 25. Mean protein levels (± S.D.) as per cent standard serum in cryptogenic cirrhosis, alcoholic cirrhosis and control subjects.

Protein	Cryptogenic cirrhosis	Alcoholic cirrhosis	Normal subjects
α_1LP	108 (28)	89 (36)	127 (20)
α_1PGP	139 (41)	140 (44)	115 (19)
α_2GP	97 (37)	93 (20)	117 (15)
α_2M	170 (51)**	171 (61)**	122 (25)
Caer	166 (69)	177 (53)**	137 (25)
10	94 (51)	118 (23)	121 (17)
Hpt	59 (84)	67 (63)	103 (44)
Hpx	104 (36)	102 (29)	107 (13)
Trf	114 (33)	91 (27)**	110 (15)
βLP	157 (38)	198 (77)	177 (43)
β_{1AC}	156 (65)**	185 (69)***	107 (24)
Albumin (g/100ml)	3.5 (0,56)	2.9 (0.42)	- -

Statistical significance of difference between mean value in clinical group and that of normal subjects

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.001$

C. Relation to biliary obstruction

i) Primary biliary cirrhosis

There were significantly reduced concentrations of prealbumin and haemopexin and increased concentrations of easily precipitable glycoprotein, α_1-antitrypsin, α_2-macroglobulin, caeruloplasmin and protein 16, (Table 26) (Fig. 26).

There were numerous correlations with the liver function tests. Haemopexin was negatively correlated with the serum bilirubin, the serum aspartate aminotransferase and the degree of prolongation of the prothrombin time ($p < 0.01$). There were negative correlations ($p < 0.01$) between the prolongation of the prothrombin time and the concentrations of easily precipitable glycoprotein, group component and protein 18. Albumin was positively correlated ($p < 0.01$) with prealbumin, haemopexin and protein 18.

There were significant ($p < 0.01$) correlations between easily precipitable glycoprotein and α_2-macroglobulin, and easily precipitable glycoprotein and protein 18.

ii) Extrahepatic biliary obstruction

There were significantly reduced concentrations of prealbumin, haemopexin, transferrin, protein 18 and β lipoprotein and increased concentrations of easily precipitable glycoprotein, α_1 antitrypsin, caeruloplasmin and protein 16 (Table 26).

Correlations with the clinical data and liver function tests included negative correlations between the concentration of transferrin and the duration of jaundice ($p < 0.01$) and the serum aspartate aminotransferase ($p < 0.01$), and haemopexin was negatively correlated with the serum cholesterol ($p < 0.01$) and serum bilirubin ($p < 0.01$).

There were significant correlations ($p < 0.01$) between the concentrations of prealbumin and group component, easily precipitable glycoprotein and

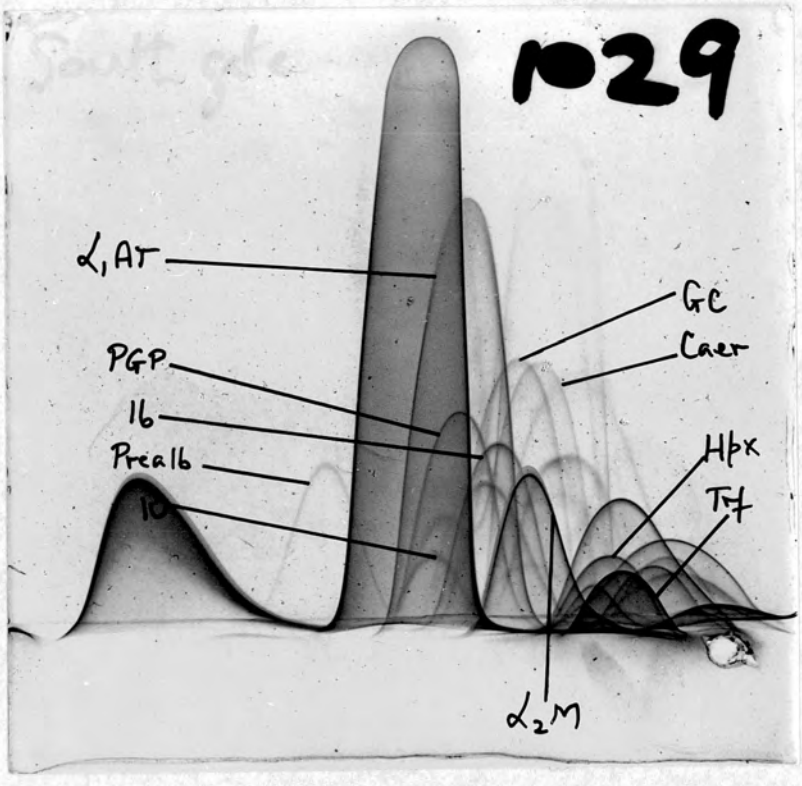


Figure 26. Serum from patient with primary biliary cirrhosis showing increased levels of α_1-antitrypsin (α_1-AT), easily precipitable glycoprotein (PGP), protein 16, caeruloplasmin (Caer), α_2-macroglobulin (α_2M), and reduced level of prealbumin (prealb).

Table 26. Mean protein levels (± S.D.) as per cent standard serum in primary biliary cirrhosis, extrahepatic biliary obstruction and control subjects.

Protein	Primary biliary cirrhosis (n=20)	Extrahepatic obstruction (n=12)	Normal subjects (n=70)
Prealb.	44.7 (18) ***	47 (22)***	111 (23)
PGP	162 (48) ***	181 (79)***	102 (15)
α_1AT	160 (39) ***	198 (75)***	99 (17)
GC	99 (58)	94 (23)	101 (15)
α_2M	169 (47) ***	140 (51)	121 (29)
Caer	210 (46) ***	230 (80)***	133 (34)
10	109 (30)	119 (41)	109 (20)
Hpt	68 (41)	102 (47)	65 (27)
Hpx	82 (37) *	58 (39)***	107 (18)
Trf	103 (42)	77 (27)**	108 (17)
+βLP	19 (6)	16 (12)**	21 (5)
16	160 (47)***	211 (117)***	103 (18)
18	106 (24)	85 (65)***	108 (13)
Albumin (g/100ml)	3.01 (0.74)	2.66 (0.65)	-

+ Expressed as % acetylated albumin

* $P < 0.05$
** $P < 0.01$
*** $P < 0.001$

protein 16, and protein 16 with both α_1-antitrypsin and protein 18.

D. Relation to iron overload

i) First series of 29 patients with primary haemochromatosis

There was significant elevation of the mean concentration of α_1-antitrypsin, group component, α_2-macroglobulin, caeruloplasmin, haemopexin and β_1AC globulin. Other proteins measured were at normal concentrations. (Table 27).

ii) Second series of haemochromatosic patients

In the further series of 20 sera from patients with primary haemochromatosis examined by the miniplate technique the only significant abnormalities were the rise in the concentration of α_2-macroglobulin and a slight reduction in prealbumin and transferrin. All other proteins were present at normal concentration (Table 27).

There were no significant correlations between the different proteins and the serum iron, serum total iron binding capacity (TIBC) or chelatable iron stores measured by the differential ferrioxamine test with the exception of transferrin which was positively correlated with the TIBC measured chemically ($N = 20$, $r = 0.753$, $p = <0.001$).

Significant correlations between pairs of proteins ($p < 0.001$) were prealbumin with group component, haemopexin with protein 18, group component with haemopexin and protein 18, haemopexin with protein 18.2

In a further series of 20 sera haemopexin alone was measured and the mean concentration (119% of the standard serum) did not differ significantly from the mean in the normal subjects.

iii) COMMENT

The striking difference in the results in the two series of patients with haemochromatosis was disturbing and totally unexpected and various possible explanations have been explored. The presence of an underlying primary hepatoma or gall bladder carcinoma was discovered in 5 cases in

Table 27. Mean protein levels (± S.D.) as per cent standard serum in two series of patients with haemochromatosis (large plates and miniplates)

Protein	Haemochromatosis (Large plates) (n=29)	Normal subjects (n=100)	Haemochromatosis (miniplates) (n=20)	Normal subjects (n=70)
Prealb.	-	-	79 (27)*	111 (23)
PGP	142 (27)	115 (19)	106 (27)	102 (15)
α_1AT	152 (39)***	114 (18)	107 (29)	99 (17)
GC	138 (41)*	117 (15)	95 (16)	101 (15)
α_2M	215 (68)***	122 (25)	193 (50)***	121 (29)
Caer	173 (59)**	137 (25)	124 (36)	133 (34)
10	-	-	116 (26)	109 (20)
Hpt	113 (71)	103 (44)	65 (34)	65 (27)
Hpx	156 (49)***	107 (13)	106 (25)	107 (18)
Trf	119 (36)	110 (15)	78 (17)**	108 (17)
βLP	146 (85)	177 (43)	†16 (6)	†21 (5)
16	-	-	102 (24)	103 (18)
18	-	-	101 (20)	108 (13)
β_1AC	155 (69)***	107 (24)	-	-
Albumin (g/100ml)	3.6 (0.64)	-	3.74 (0.88)	-

† Expressed as % acetylated albumin

* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

the first series up to 3 years after the serum was examined (cases 5, 10, 13, 17 and 18). It is known that raised haemopexin levels may occur in malignant melanoma (Manuel et al, 1970) and a variety of other tumours (Muller and Muller von Voigt, 1967). However the haemopexin levels in our cases with malignant disease were 230, 186, 124, 183, 199% of the standard and the mean of this group (168%) does not differ from the remainder (167%). The other patients remain well 4 years later apart from 4 who have died (Cases 2 and 21 congestive cardiac failure, Case 24 cerebrovascular accident, Case 15 peripheral vascular disease).

The high haemopexin levels could not be accounted for by gross iron overload before treatment or diabetes mellitus (Cleve et al, 1968). Samples taken at the end of a venesection session might have raised level of most proteins due to gross congestion in the limb. However there was no relation between raised haemopexin levels and current venesection therapy. Further fresh samples taken from 12 of these patients were examined 3½ to 4 years later by the miniplate technique and except for Case 10 all were within the normal range.

It is noteworthy that haemopexin is but one of several plasma proteins raised in the sera of patients in the first series. Review of the plates showed that there was no error in the planometric measurement but it seems possible that the measurement of the serum sample or the acetylated albumin was at fault. This would account for a general rise in the concentration of all proteins on the plate and is supported by the finding that plates with normal haemopexin levels had normal concentrations of most other proteins. The number of proteins apart from haemopexin present on each plate at a concentration above the normal range (mean + 2 S.D.) correlated closely with the haemopexin level ($n = 29$; $r = 0.755$; $p < 0.001$). This is strong evidence that an artefact in measuring the serum volume or acetylated albumin was responsible for the findings in this first series

of patients with haemochromatosis.

The findings in the second series of patients with a rise in α_2 macroglobulin and a slight fall in prealbumin and transferrin are more in keeping with the findings in the other groups of patients with liver disease. This minor abnormality is consistent with the low grade disease process and the excellent liver function in these patients, and is closely similar, as might be expected, to the findings in the patients with inactive cryptogenic cirrhosis.

iv) Effects of serial venesection in six patients

The initial serum concentrations of the 13 proteins measured are shown in Table 28. There was little change in the concentration of any protein over the next few months in the course of the venesection in any patient, with the exception of the transferrin concentration which was below the normal range in 2 patients and at the lower end of normal in 1 other before venesection started. During venesection there was a progressive rise in concentration in these 3 patients. Representative readings in patient 6 are shown in Fig. 27. Consistently low levels of α_1-antitrypsin were noted in this patient and starch gel electrophoresis (Dr. P. Cook) showed heterozygous deficiency associated with Pi phenotype MZ.

v) Relatives of patients with primary haemochromatosis

The mean serum haemopexin concentration was 123 $\pm$ S.D. 22% of the standard and did not differ significantly from the concentration in the normal population. There was no correlation with the serum iron, TIBC or iron stores. The mean serum transferrin concentration was 102 $\pm$ S.D. 13.5% standard and this was significantly lower ($p < 0.01$) than the control population. There was no correlation with the serum iron or chelatable iron stores.

Table 28. Protein concentrations as per cent standard serum in six patients with primary haemochromatosis before the start of venesection therapy.

Protein	Patient Number 1	2	3	4	5	6	Normal Range (Mean ± 2 S.D.) (n=70)
Prealb	72	110	143	49	87	79	65 - 158
α_1PGP	106	64	78	120	104	125	72 - 134
α_1AT	97	121	97	135	85	54	64 - 133
GC	83	127	107	81	93	97	70 - 132
α_2M	210	150	173	261	195	205	63 - 178
Caer	114	160	88	144	95	156	64 - 203
10	94	139	172	134	139	129	68 - 151
Hpt	106	66	35	15	70	90	10 - 120
Hpx	112	140	140	90	117	114	70 - 144
Trf	50	62	108	90	77	82	75 - 143
*βLP	15	23	17	14	19	14	11 - 42
16	93	146	66	100	94	112	67 - 140
18	95	136	89	99	97	114	81 - 137

* expressed as percentage of area of acetylated albumin

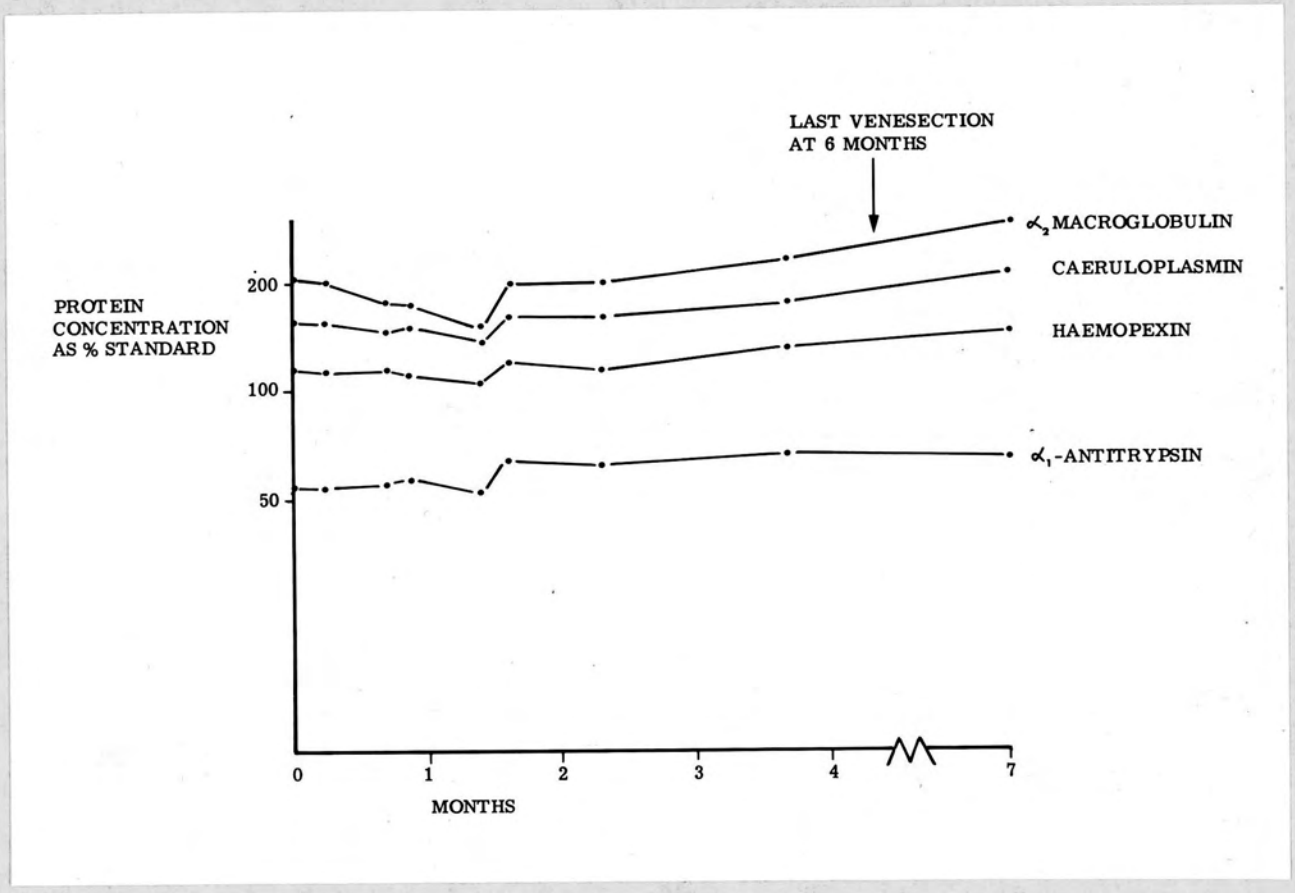


Figure 27. Concentration of four proteins at intervals during regular venesection therapy in a patient with primary haemochromatosis.

vi) Patients with secondary iron overload

The mean serum haemopexin concentration was 113.8 ± S.D. 27% of the standard and this did not differ significantly from the control population.

E. Evidence of in vivo complement activation

Slight conversion of β_1C was seen in fresh plasma in 2 of the 3 normal subjects (Fig.28), all 6 of the patients with active chronic hepatitis and one of 3 patients with primary biliary cirrhosis. The degree of conversion in each case was slight and no greater than that noted in the control subjects.

F. Change in protein pattern following liver transplantation

Case 1. (Appendix D). The concentrations of some of the 18 proteins measured before transplanation are shown in Table 29 and the levels of all except protein 10 are raised. The changes in serum protein concentrations observed after transplantation (Fig.29) led to the definition of 2 principal groups. Group (A) included those proteins which stayed at normal or increased concentrations from the time of transplantation till death. These included easily precipitable glycoprotein, α_1-antitrypsin, group component α_2-macroglobulin, caeruloplasmin and haptoglobin. Levels increased in the second post-operative week during the early rejection episode but were normal by the 56th day when the concentrations of all the proteins measured were in the normal range. Levels then increased again during the phase of chronic rejection. Group (B) included those that gradually decreased in concentration after the transplant, particularly after the 80th day. They included albumin, protein 10, transferrin and haemopexin. The concentration of β_1A-C globulin (C3) decreased in the first 17 days at the time of the early acute rejection episode (Fig.29). At the beginning of the period of chronic rejection the concentration was also greatly depressed but later when infection may have been an important stimulus to synthesis it

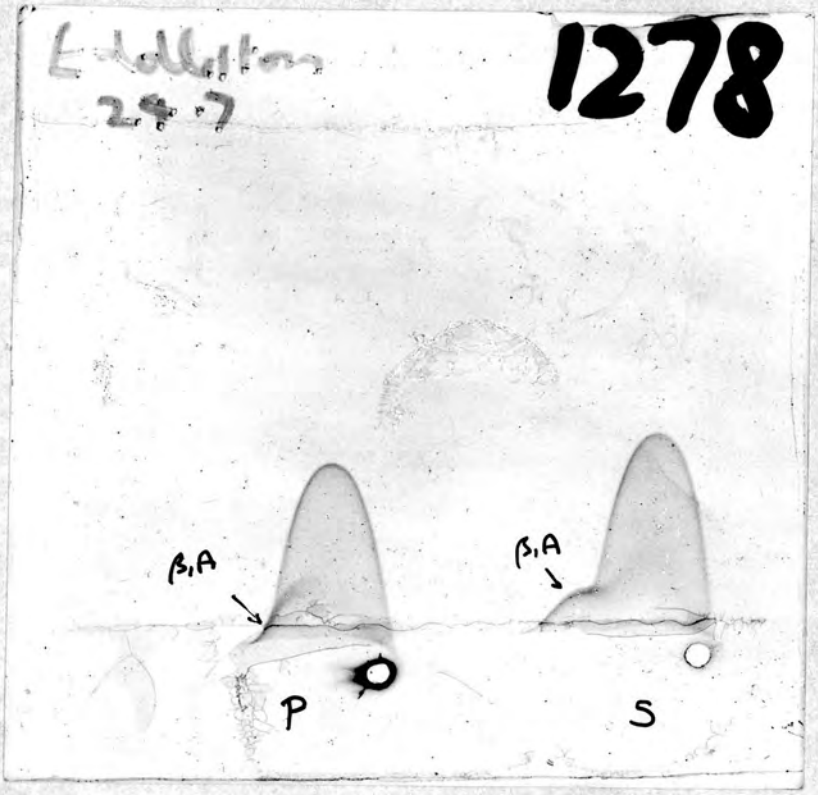


Figure 28. Two dimensional immunoelectrophoresis pattern using antiserum to β_1AC with fresh plasma (left) and serum (right) from a normal subject. Both β_1C and β_1A are seen in both specimens.

Table 29. Protein concentration as percent standard serum in 4 patients prior to orthotopic liver transplantation

Protein	CASE NUMBER 1†	2	3	4	Normal Range (Mean ± 2 S.D.)
Prealb.	-	17	52	50	65 - 158
α_1PGP	218	35	122	162	72 - 134
α_1AT	236	97	157	214	64 - 133
GC	188	80	99	111	70 - 132
α_2M	183	123	114	146	63 - 178
Caer.	300	56	178	195	64 - 203
10	98	71	67	104	68 - 151
Hpt	565	0	137	140	10 - 120
Hpx	177	24	119	146	70 - 144
Trf	178	50	58	108	75 - 143
*βLP	-	-	18	20	11 - 42
16	-	58	235	241	67 - 140
18	-	-	70	128	81 - 137

* Expressed as percentage of area of acetylated albumin

† Standard reference serum was National Institute for Medical Research 67/86.

increased to normal. Prealbumin decreased initially at the time of the marked increase in acute phase proteins and again during the second increase in acute phase proteins after the 80th day.

Case 2. (Appendix D) Pre-operatively the concentrations of most proteins were low but the levels of α_1-antitrypsin, group component and α_2-macroglobulin were within the normal range (Table 29). Following transplantation the concentrations of α_1-antitrypsin and protein 16 rose to supranormal levels and easily precipitable glycoprotein, caeruloplasmin, haptoglobin, and haemopexin rose to normal (Fig.30). Prealbumin and transferrin rose slightly but remained low. Most proteins were at maximal concentration on days 3 and 4 and fell slightly over days 5 and 6 when liver function was deteriorating due to acute rejection of the graft.

Case 3. Before transplantation levels of α_1-antitrypsin and protein 16 were raised and prealbumin and transferrin reduced (Table 29). Following transplantation (Fig.31) prealbumin and transferrin rose to normal and α_1-antitrypsin gradually fell. Protein 16 remained high and there was also a rise in haemopexin, protein 10, easily precipitable glycoprotein, and α_2-macroglobulin to above the normal range in the second and third months. Apart from α_1-antitrypsin which rose again there was little change in the concentration of other proteins. The last sample was taken when the patient was weak and cachectic 2 months before her death from carcinomatosis. At this time liver function tests were entirely normal and the concentrations of easily precipitable glycoprotein, α_1-antitrypsin, $\alpha_2$macroglobulin, haemopexin and protein 16 were above the normal range. The concentrations of the other proteins measured were normal.

Case 4. Before transplantation levels of easily precipitable glycoprotein, α_1-antitrypsin, haptoglobin and protein 16 were high and prealbumin was low (Table 29). Following transplantation haptoglobin fell

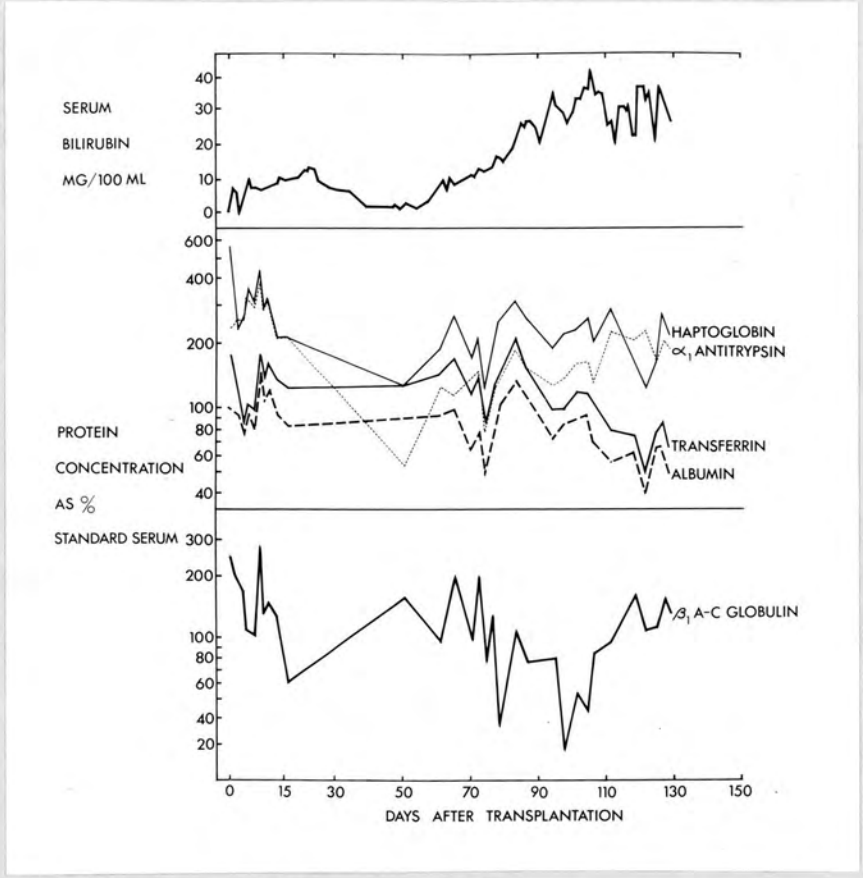


Figure 29. Case 1. Serial concentrations of five proteins at intervals after orthotopic transplantation of the liver.

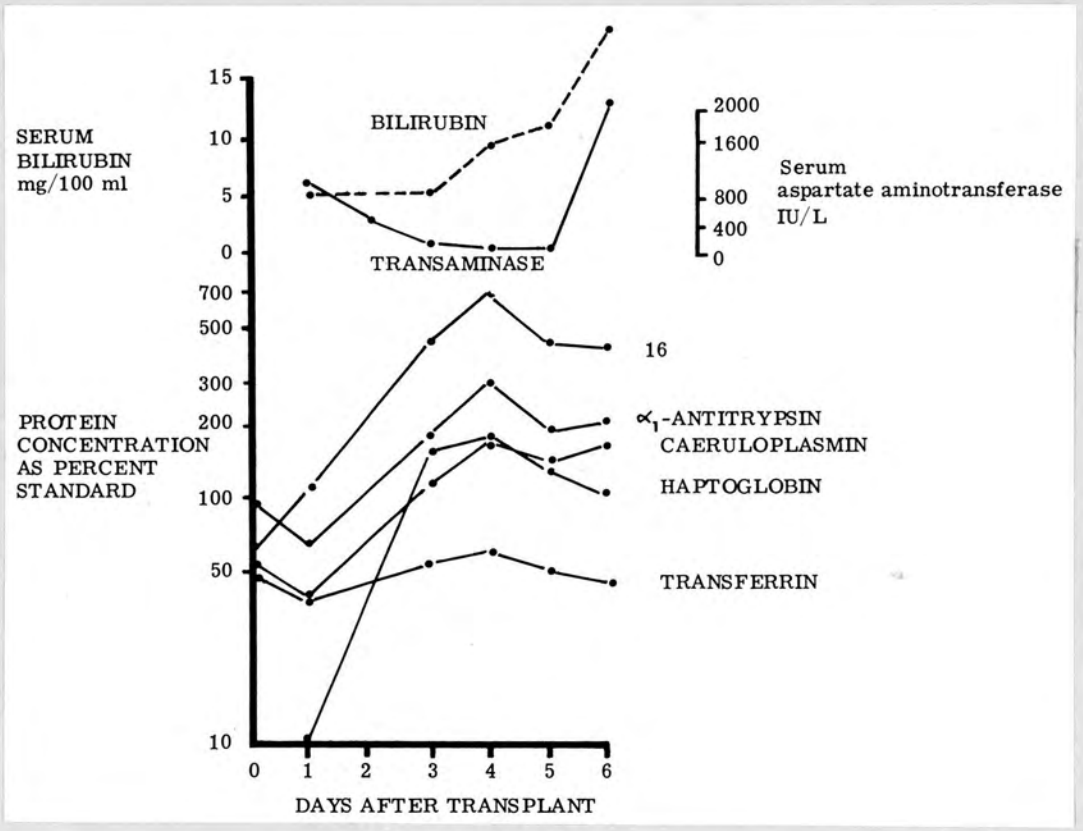


Figure 30. Case 2. Serial concentration of five proteins at intervals after orthotopic transplantation.

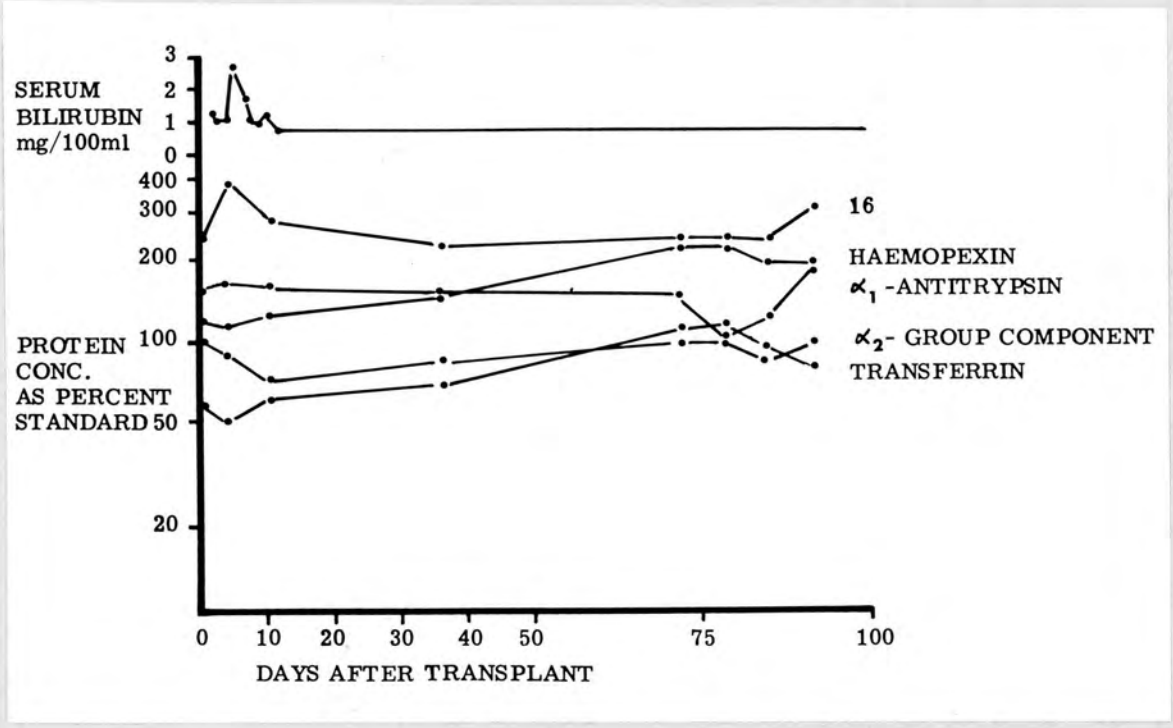


Figure 31. Case 3. Concentration of five proteins at intervals after orthotopic transplantation of the liver.

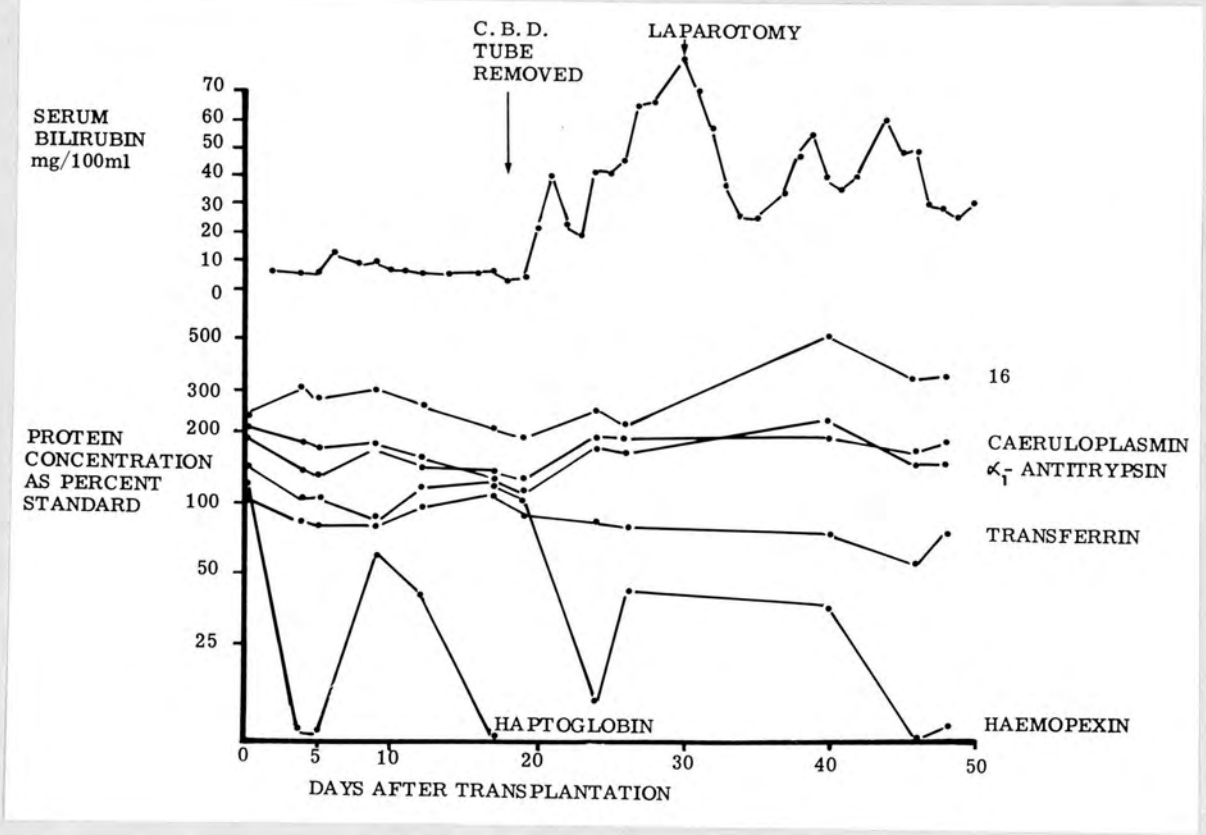


Figure 32. Case 4. Concentration of six proteins at intervals after orthotopic transplantation of the liver.

and after the 17th day it remained undetectable (Fig.32). On the 19th day when liver function was at its best the protein pattern was normal apart from absence of haptoglobin and persistent elevation of protein 16. Thereafter in association with the progressive obstructive jaundice the protein pattern became increasingly abnormal with raised levels of easily precipitable glycoprotein, α_1-antitrypsin and protein 16. Levels of group component, α_2-macroglobulin, caeruloplasmin, protein 10, β lipoprotein and protein 18 remained normal but haemopexin and transferrin fell below the normal range, the former being undetectable in one specimen.

G. Plasma protein pattern in patients with α_1-antitrypsin deficiency

Apart from the strikingly low level of α_1 antitrypsin (Fig.11) the main change was a rise in the mean level of α_2 macroglobulin (Table 30) due to levels of 215 and 356% in two patients. Concentrations were within the normal range in the other 5 patients. There was a slight rise in protein 10 but the concentration of other proteins measured were normal.

H. Influence of hepatic microsomal enzyme induction

i) Normal Subjects

Levels of all plasma proteins measured were within the normal range in both subjects before treatment. The 5 day course of bucolome (Butymidin, Takeda Chemical Industries Ltd., Japan) 600mg b.d. did not result in a significant change (Figs.34, 34). However there was probably no significant induction of hepatic enzumes for there was no rise in urinary D-glucaric acid excretion and the fall in plasma bilirubin in one subject was slight. On the other hand the seven day course of N-phenylphetharbital (phetharbital) 200mg t.d.s. was accompanied by a fall in plasma bilirubin and a rise in D-glucaric acid excretion in both subjects but in neither subject was there a change in the concentration of any of the plasma proteins measured (Figs.33, 34).

Table 30. Mean protein levels (± S.D.) as percent standard serum in seven patients with homozygous α_1AT deficiency

Protein	α_1AT deficiency subjects (n=7)	Normal subjects (n=70)
Prealb	106 (23)	111 (23)
PGP	112 (9)	102 (15)
α_1AT	20.9 (6.8)***	99 (17)
GC	100 (21)	101 (15)
α_2M	186 (80)***	121 (29)
Caer.	150 (34)	133 (34)
10	150 (28)**	109 (20)
Hpt	88 (32)	65 (27)
Hpx	109 (18)	107 (18)
Trf	109 (25)	108 (17)
†β_{LP}	25 (6)	21 (5)
16	105 (17)	103 (18)
18	106 (15)	108 (13)

† Expressed as % acetylated albumin

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

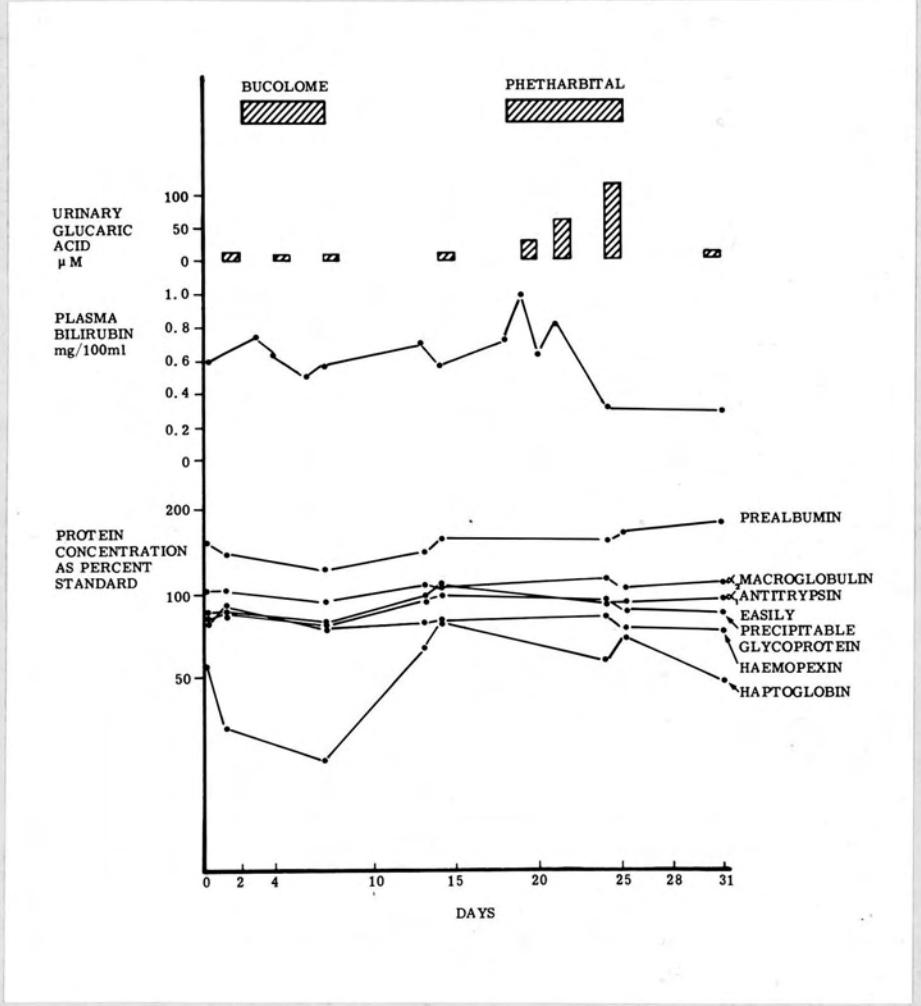


Figure 33. Serial concentrations of six proteins in a normal subject during induction of hepatic microsomal enzymes with phetharbital. Note the rise in urinary glucaric acid excretion and the fall in plasma bilirubin.

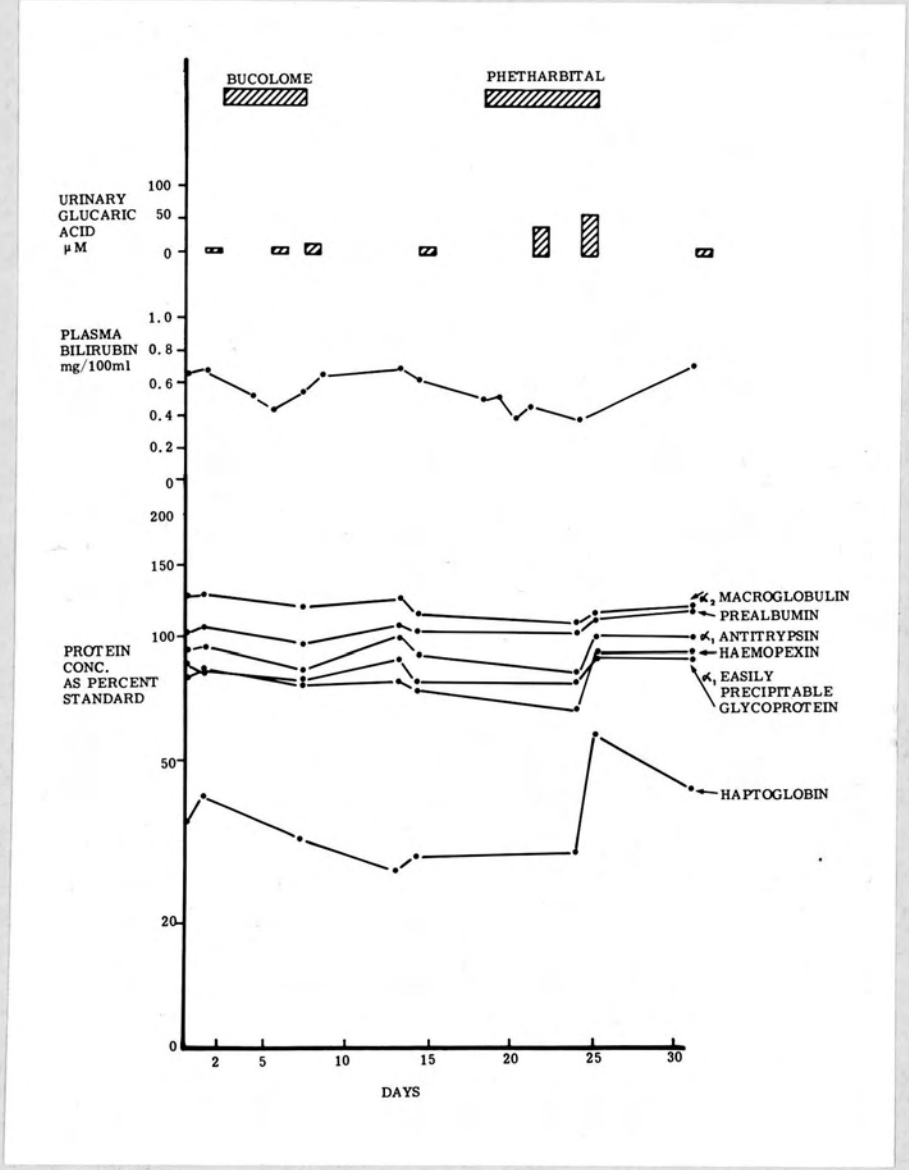


Figure 34. Serial concentrations of six proteins in a normal subject during induction of hepatic microsomal enzymes with phetharbital. Note the rise in urinary glucaric acid excretion and the fall in plasma bilirubin.

ii) Epileptics

The mean level of α_1-antitrypsin, group component, α_2-macroglobulin, caeruloplasmin and protein 16 were all significantly ($p<0.001$) raised above control values (Table 31).

The D-glucaric acid excretion was raised in 13 of the 14 patients in whom it was measured (Table 17) and there was a weak positive correlation between the glucaric acid excretion and the number of units of anticonvulsant taken ($r = 0.611$; $t = 2.72$; $p<0.02>0.01$). There was no significant correlation between the serum calcium, alkaline phosphatase, D-glucaric acid excretion or number of units of anticonvulsant taken and the concentration of any of the proteins measured. There were significant correlations ($p<0.01$) between easily precipitable glycoprotein and α_1-antitrypsin, protein 10 and protein 18 and between protein 16 and α_1-antitrypsin and caeruloplasmin.

I. Comparison of protein concentrations in different disease groups

The mean concentration of each protein in seven of the diagnostic groups examined (viral hepatitis, active chronic hepatitis, primary biliary cirrhosis, extrahepatic obstruction, alcoholic cirrhosis, cryptogenic cirrhosis and haemochromatosis) was compared with the concentration in the other groups in addition to the control population (Table 32). As the reference standard serum and control population for the miniplate studies were different from those used in the initial large plate studies, comparisons were made only between diagnostic groups examined by the same technique. The significance of the differences between the means of the groups was calculated from the overall F value by the Newman-Keul's method (Winer, 1962). As Fisher's F value from the analysis of variance was greater for the logged protein values, only logged data was used. When the mean protein values for the three groups of subjects studied on large plates (cryptogenic and alcoholic cirrhosis

Table 32. Protein concentrations mean (± S.D.) expressed as % standard in seven diagnostic groups and control subjects.

Protein	MINI PLATE STUDIES					LARGE PLATE STUDIES			
	Hepatitis (n=12)	Extrahepatic obstruction (n=12)	Primary biliary cirrhosis (n=20)	Primary Haemochromatosis (n=20)	Normal subjects (n=70)	Cryptogenic cirrhosis (n=12)	Alcoholic cirrhosis (n=12)	Active chronic hepatitis (n=20)	Normal subjects (n=100)
Prealb	56(40)***	47(22)***	44.7(18)***	79(27)*	111(23)	-	-	-	-
PGP	182(44)***	181(79)***	162(48)***	106(27)	102(15)	139(41)	140(44)	151(42)***	115(19)
α_1AT	136(20)**	198(75)***	160(39)***	107(29)	99(17)	-	-	-	114(18)
GC	91(19)	94(23)	99(58)	95(16)	101(15)	97(37)	93(20)	105(35)	117(15)
α_2M	135(41)	140(51)	169(47)***	193(50)***	121(29)	170(51)**	171(61)**	175(51)***	122(25)
Caer	179(67)*	230(80)***	210(46)***	124(36)	133(34)	166(69)	177(53)**	175(45)***	137(25)
10	170(55)***	119(41)	109(30)	116(26)	109(20)	94(51)	118(23)	143(57)	121(17)
Hpt	56(46)*	102(47)	68(41)	65(34)	65(27)	59(84)	67(63)	48(52)**	103(44)
Hpx	58(38)***	58(39)***	82(37)*	106(25)	107(18)	104(36)	102(29)	108(38)	107(13)
Trf	98(22)	77(27)**	103(42)	78(17)**	108(17)	144(33)	91(27)**	135(39)**	110(15)
βLP	+34(11)*	+16(12)**	+19(6)	+16(6)	+22(5)	157(38)	198(77)	175(67)	177(43)
16	131(32)	211(117)***	160(47)***	102(24)	103(18)	-	-	-	-
18	112(33)	85(65)***	106(24)	101(20)	108(13)	-	-	-	-
β_1AC	-	-	-	-	-	156(65)**	185(69)***	185(87)**	107(24)
Albumin (g/100ml)	3.46(0.46)	2.66(0.65)	3.01(0.74)	3.74(0.88)	-	3.5(0.56)	2.9(0.42)	3.2(0.66)	-

+ expressed as % acetylated albumin

* $P < 0.05$
** $P < 0.01$
*** $P < 0.001$

and active chronic hepatitis) were compared with the values from the miniplate control population rather than the original large plate control population, many of the significant differences were no longer found. This is probably attributable to differences between the standard reference sera used for the large and small plate studies and no attempt was therefore made to compare the results of immunoelectrophoresis in patient groups studied on large and small plates.

Prealbumin. There was no significant difference between the levels in hepatitis, primary biliary cirrhosis or extrahepatic obstruction but the levels in each disease was significantly lower than in haemochromatosis ($p < 0.001$).

Easily precipitable glycoprotein. There was no significant difference between the levels in hepatitis, primary biliary cirrhosis or extrahepatic obstruction but the level in each of these diseases was significantly higher than in haemochromatosis ($p < 0.001$). There was no significant difference between the cryptogenic cirrhosis, alcoholic cirrhosis or active chronic hepatitis groups.

α_1-antitrypsin. The concentration in hepatitis was significantly less than in extrahepatic obstruction ($p < 0.05$) but higher than in haemochromatosis ($p < 0.05$). There was no difference between the mean level in primary biliary cirrhosis or extrahepatic obstruction but the level in both these diseases was significantly higher than in haemochromatosis ($p < 0.001$).

Group component. There was no difference in concentration between any of the seven diagnostic groups.

α_2-macroglobulin. The levels in haemochromatosis were significantly higher than in hepatitis and extrahepatic obstruction ($p < 0.005$) but there was no difference between haemochromatosis and primary biliary cirrhosis ($p < 0.05$). There was no difference between the levels in cryptogenic cirrhosis, alcoholic cirrhosis or active chronic hepatitis.

Caeruloplasmin. The levels were significantly higher in primary biliary cirrhosis and extrahepatic obstruction ($p < 0.001$) and hepatitis ($p < 0.01$) than in haemochromatosis and the levels in extrahepatic obstruction were greater than in hepatitis ($p < 0.05$). There were no differences between the three groups studied on large plates.

Protein 10. The levels in hepatitis were significantly greater than in primary biliary cirrhosis, haemochromatosis ($p < 0.001$) and extrahepatic obstruction ($p < 0.005$). There was no difference between the levels in cryptogenic and alcoholic cirrhosis and active chronic hepatitis.

Haptoglobin. The mean concentration in hepatitis was significantly lower than in extrahepatic obstruction ($p < 0.005$) and haemochromatosis ($p < 0.05$). There was no difference in the concentration in cryptogenic and alcoholic cirrhosis and active chronic hepatitis.

Haemopexin. The mean concentration in hepatitis and extrahepatic obstruction was significantly lower than in primary biliary cirrhosis ($p < 0.05$) and haemochromatosis ($p < 0.001$) and the level in primary biliary cirrhosis was lower than in haemochromatosis ($p < 0.05$). There was no significant difference between the levels in cryptogenic and alcoholic cirrhosis and active chronic hepatitis.

Transferrin. The mean level in extrahepatic obstruction was lower than in primary biliary cirrhosis and hepatitis ($p < 0.05$) and that in alcoholic cirrhosis was lower than in active chronic hepatitis ($p < 0.001$).

β-lipoprotein. The mean level in hepatitis was significantly greater than in extrahepatic obstruction and haemochromatosis ($p < 0.001$) and primary biliary cirrhosis ($p < 0.005$). There were no differences in the levels in cryptogenic and alcoholic cirrhosis and active chronic hepatitis.

Protein 16. The mean level in primary biliary cirrhosis and extrahepatic obstruction was significantly greater than in haemochromatosis ($p < 0.001$) and hepatitis ($p < 0.01$).

Protein 18. The mean level in extrahepatic obstruction was significantly lower ($p < 0.001$) than in primary biliary cirrhosis, haemochromatosis and hepatitis.

β_1AC globulin (C3). There was no significant difference between the concentration in cryptogenic and alcoholic cirrhosis and active chronic hepatitis.

J. Diagnostic value of the plasma protein concentrations studied by discriminant analysis. Comparison with cellulose acetate electrophoresis

A sequential Bayesian function and a stepwise discriminant function based on the logged values of 13 proteins in patients studied by the miniplate method were used to reclassify 134 patients into their 5 diagnostic groups (controls, primary biliary cirrhosis (PBC), extrahepatic obstruction (E.H.Obs.), Viral hepatitis (Hep), and primary haemochromatosis (Haem)). Because the data from the large plate studies were not strictly comparable with the miniplate studies (see above) the groups with active chronic hepatitis (ACH), alcoholic cirrhosis (Alc) and cryptogenic cirrhosis (Crypt) have been analysed separately.

(i) Immunoelectrophoresis results studied by sequential Bayesian function. The results of reclassification of the 134 cases according to this function are shown in Table 33. The overall error was 12%, and the errors for the individual groups are given. The order of variables selected by the computer and the average reduction of uncertainty of the diagnostic group is also shown.

Reclassification of the patients studied on large plates into their 3 diagnostic groups was achieved with an error rate of 34%. No control group was included and values for 8 proteins only were used (Table 34). Reclassifying the miniplate patients using the same eight proteins was achieved with an error of 17% (Table 35).

Table 33. Reclassification of 134 patients into the five diagnostic groups using a Bayesian function based on the logged values of the concentrations of the 13 proteins measured.

True disease	Calculated disease groups						
	Controls	P.B.C.	E.H.Obs.	Hep.	Haem.	Errors	Errors %
Controls	62	1	0	0	7	8/70	11
P.B.C.	0	20	0	0	0	0/20	0
E.H.Obs.	0	2	9	1	0	3/12	25
Hep.	0	2	0	10	0	2/12	17
Haem	1	1	1	0	17	3/20	15

Overall error in reclassification 16/134 = 12%

Order of the variables chosen and average reduction of uncertainty of the diagnosis.

Hpt	.24	18	.16
Prealb	.24	Caer	.13
βLP	.21	Trf	.12
16	.19	10	.07
PGP	.18	α_2M	.05
Hpx	.17	GC	.04
α_1AT	.17		

Table 34. Reclassification of 41 patients into three diagnostic groups using a Bayesian function based on the logged values of the concentrations of the 8 proteins measured.

True disease	Calculated disease groups				
	Crypt	Alc	ACH	Error	Error %
Crypt	4	0	7	7/11	64
Alc	0	6	4	4/10	40
ACH	1	2	17	3/20	15

Overall error in reclassification 14/41 = 34%

Order of the variables chosen and average reduction of uncertainty of the diagnosis.

Trf	.2	Caer	.04
10	.13	$_2$M	.03
PGPO	.05	Hpt	.02
GC	.04	Hpx	.01

Table 35. Reclassification of 134 patients into five diagnostic groups using a Bayesian function based on the logged values of the concentrations of 8 proteins.

True disease	Calculated disease groups						
	Controls	P.B.C.	E.H.Obs.	Hep.	Haem	Error	Error %
Controls	63	2	1	0	4	7/70	10
P.B.C.	1	14	4	1	0	6/20	30
E.H.Obs.	0	2	7	2	1	5/12	42
Hep	0	2	0	10	0	2/12	17
Haem	1	1	0	1	17	3/20	15

Overall error in reclassification 23/134 = 17%

Order of the variables chosen and average reduction of uncertainty of the diagnosis.

PGP	.29	Caer	.13
Hpt	.28	α_2M	.12
Hpx	.2	10	.07
Trf	.14	GC	.04

(ii) Immunoelectrophoresis results studied by stepwise linear discriminant function.

Reclassification of the 134 patients into the 5 diagnostic groups based on this function is shown in Table 36. The overall error in reclassification using the group of 8 proteins was 19.1% and the individual errors and the rank order of the variables in reducing the diagnostic uncertainty are shown. The error in reclassifying 41 patients with cryptogenic, alcoholic cirrhosis and active chronic hepatitis using 8 proteins was 31%.

(iii) Cellulose acetate electrophoresis results

There were data available for 85 patients in 7 diagnostic groups. Seven readings were used from each cellulose acetate strip (total protein, albumin, total globulin, α_1, α_2, β and γ globulins). Using a stepwise linear discriminant function the overall error in reclassification was 51% (Table 37).

Table 37. Error in reclassifying 85 patients into seven diagnostic groups using a stepwise discriminant function based on the 7 logged values derived from the cellulose acetate electrophoresis strips.

Diagnostic group	Error	Error %
Cryptogenic cirrhosis	6/11	54
Alcoholic cirrhosis	6/10	60
1° Haemochromatosis	6/10	60
Chronic active hepatitis	8/20	40
Acute hepatitis	6/11	54
E.H. Obstruction	3/8	38
1° biliary cirrhosis	8/15	53
Overall error	43/85	51

Table 36. Reclassification of 134 patients into the five diagnostic groups using a stepwise discriminant function based on the logged values of the concentrations of 8 proteins.

True disease	Calculated disease groups						
	Controls	P.B.C.	E.H.Obs.	Hep.	Haem.	Errors	Errors %
Controls	62	2	0	0	6	8/70	11.4
P.B.C.	2	12	3	2	1	8/20	40
E.H.Obs.	2	0	7	2	1	5/12	42
Hep.	1	1	0	9	1	3/12	25
Haem.	2	0	0	0	18	2/20	10

Overall error in reclassification 26/134 = 19.4%

Rank order of the variables in reducing the diagnostic uncertainty.

PGP	10
α_2M	Trf
Hpx	Hpt
Caer	GC

When only 44 patients with 4 diseases were considered (Haemochromatosis, viral hepatitis, extrahepatic obstruction and primary biliary cirrhosis) the error in reclassification was 36% (Table 38). This was greater than the error in reclassifying the same 44 patients on the basis of 8 proteins values derived from immunoelectrophoresis, the combination of those 8 values together with the 7 cellulose acetate electrophoresis values, the 13 proteins derived from immunoelectrophoresis, and the total group of 20 values derived from both immunoelectrophoresis and cellulose acetate electrophoresis. Each patient was assigned to the correct diagnostic group when all 20 protein values were used (table 38). The equivalent values using a sequential Bayesian function are also shown. The rank order of the variables according to their importance in reducing the diagnostic uncertainty is given in Table 39, both for the group of 13 proteins and the group of 20 values.

The error in reclassifying 41 patients with alcoholic and cryptogenic cirrhosis and active chronic hepatitis is shown in Table 40.

Table 38. Percent error in reclassification of 44 patients with 4 diseases using a sequential Bayesian function and a stepwise linear discriminant function based on logged data derived from immunoelectrophoresis and cellulose acetate. strip.

Protein data used in analysis	Bayesian function % error	Linear discriminant function % error
7 values from cellulose acetate strip	37	36
8 proteins from immunoelectrophoresis	29.5	23
15 values above	18	4.5
13 proteins from immunoelectrophoresis	16	23
20 values from cellulose acetate strip and immunoelectrophoresis	9.1	0

Table 39. Rank order of variables in reducing the diagnostic uncertainty for 44 patients with 4 diseases analysed by a stepwise linear discriminant function.

	13 proteins from immunoelectrophoresis	13 proteins + 7 values from cellulose acetate electrophoresis
1	Protein 16	16
2	βLP	βLP
3	α_2M	α_2M
4	Caer	Caer
5	PGP	Total protein
6	Protein 10	Protein 10
7	Prealb.	Hpt
8	Hpx	Trf
9	Trf	γ globulin
10	Protein 18	α_1 globulin
11	α_1AT	PGP
12	Hpt	α_2 globulin
13	GC	Albumin
14		β globulin
15		Total globulin
16		GC
17		Protein 18
18		Hpx
19		α_1AT
20		Prealb.

Table 40. Percent error in reclassification of 41 patients with cryptogenic cirrhosis (11), alcoholic cirrhosis (10) and active chronic hepatitis (20) using a sequential Bayesian function and a stepwise linear discriminant function based on logged data derived from immunoelectrophoresis and cellulose acetate electrophoresis.

Protein data used in analysis	Bayesian function % error	Linear discriminant function % error
7 values from cellulose acetate strip	24	24
8 proteins from immunoelectrophoresis	34	31
15 values above	12	9.7

Table 31. Mean protein level (± S.D.) as percent standard serum in 15 epileptic patients on long term anticonvulsant therapy.

Protein	Epileptics (n=15)	Normal Subjects (n=70)
Prealb.	126 (31)	111 (23)
PGP	93 (15)*	102 (15)
α_1AT	132 (22)***	99 (17)
GC	130 (17)***	101 (15)
α_2M	175 (48)***	121 (29)
Caer.	180 (33)***	133 (34)
10	104 (24)	109 (20)
Hpt	83 (45)	65 (27)
Hpx	115 (17)	107 (18)
Trf	94 (19)	108 (17)
†βLP	25 (11)	21 (5)
16	139 (33)***	103 (18)
18	111 (17)	108 (13)

+ Expressed as % acetylated albumin

* $p < 0.05$ ** $p < 0.001$ *** $p < 0.001$

PART 6

DISCUSSION AND WORK FOR THE FUTURE

DISCUSSION

The pattern of the changes in the plasma protein concentrations is very similar in each of the liver diseases studied, although the degree of abnormality is variable. The changes are least marked in the patients with haemochromatosis and cryptogenic cirrhosis, these being the two groups studied with the least disturbance of liver function. The relationship between the protein changes and the degree of disturbance of liver function is strengthened both by finding significant correlations in most patient groups between different parameters of liver function and individual plasma proteins, and the serial studies on the patients with active chronic hepatitis and liver allografts which, in general, showed that as liver function improved the concentration of many of the proteins returned to normal. However, even with entirely normal liver function tests, if there was underlying chronic liver disease, some proteins tended to remain at abnormal concentrations.

Relationship of changes in plasma proteins to the "acute phase reaction".

The terms "acute phase reaction" is used to describe the changes in the concentration of several serum proteins in response to tissue injury. The stimuli responsible for these changes include surgical trauma (Werner and Cohnen 1969; Clarke et al, 1971) myocardial infarction, infection and neoplasia (Muller and Muller von Voigt, 1968 a, b). The changes in the plasma protein pattern are monotonously predictable and in no way diagnostic for the responsible underlying disease process.

The changes are perhaps best illustrated by considering the results of surgical trauma. Detailed studies have been made of the concentrations of several plasma proteins before and for 2 to 3 weeks following a variety of types of operation (Werner and Odenthal 1967; Crockson et al, 1966; Werner and Cohnen, 1969; Clarke et al, 1971; Aronsen et al, 1972). The

results agree closely although there is some indication that the degree of disturbance of plasma proteins is proportional to the extent of the surgery. The concentrations of C-reactive protein, orosomucoid, haptoglobin and α_1-antitrypsin rise rapidly to a maximum at 3-4 days and there is a smaller and less marked rise in the concentrations of haemopexin, caeruloplasmin, C3 and fibrinogen at the end of the first post-operative week. In their study on patients after hernia repair Clarke et al, (1971) also noted a rise in group component, easily precipitable glycoprotein, and protein 16. There is a consistent fall in albumin, α_1 lipoprotein, prealbumin and transferrin. Levels of α_2-macroglobulin IgM and IgG show little change. The protein pattern has usually returned to normal by 14-18 days post-operatively unless infection prolongs the stimulus for the acute phase reaction (Werner and Cohnen, 1969).

Since the introduction of immunological methods of determining individual plasma proteins, a wide variety of disease states have been studied and although there are minor differences, the basic pattern outlined above is found to hold true. Examples include myocardial infarction (Muller and Muller von Voigt, 1968b; Johansson, et al, 1972; Agostini, et al, 1970) ulcerative colitis and Crohn's disease (Weeke and Jarnum, 1971) rheumatoid arthritis (Muller and Muller von Voigt, 1968b; Clarke et al, 1970b) tuberculosis (Muller and Muller von Voigt, 1968a; Clarke et al, 1970a) and a wide variety of malignant diseases (Muller and Muller von Voigt, 1968a, b.).

Understanding of the mechanisms involved in the expression of the acute phase reaction has advanced in recent years. The fall in albumin after surgery is attributable in part to loss into the wound site (Mouridsen, 1967) but in experimental induction of the acute phase reaction by chemical irritants albumin synthesis has been shown to be reduced

(Gordon, 1970). A reduction in synthesis is also thought to account for the fall in prealbumin concentration (Bernstein, et al, 1967). Increased catabolism may also play a part and is a major factor determining the fall in transferrin at least in infections (Jarnum and Lassen, 1961). The rise in globulins was at first attributed to release of protein from the ground substance of connective tissue (Bole and Leutz, 1967) but the reduction in the response which was noted to occur in man when partial hepatectomy was performed (Wollheim et al, 1969) suggests rather that the liver may be the site of increased synthesis. This effect of partial hepatectomy in reducing the acute phase response had been shown previously in the rat by Darcy (1965) who studied a specific acute phase $\alpha_1$globulin (α_1AP globulin) later shown to be produced in the liver (Weimer, et al, 1965). The hepatic synthesis of another acute phase rat globulin (α_2AP) was shown in perfusion experiments to increase tenfold after injury (Sarcione, 1970) and there is similar evidence (Miller and John, 1970) that increased synthesis of other proteins accounts for the rise in plasma concentration in the acute phase reaction. Further evidence for the importance of de novo synthesis of acute phase proteins comes from the effects of actinomycin D and other inhibitors of protein synthesis which prevent the rise in haptoglobin and other acute phase globulins usually seen after laparotomy or subcutaneous injection of turpentine in rats (Neuhauseet al, 1966; Maung et al, 1968; Sarcione, 1970).

The nature of the signal which increases hepatic synthesis of some proteins and decreases that of others is not known and experiments in which rats livers were perfused with blood from injured animals have not given conclusive evidence for a circulating factor (Gordon and Koj, 1968; Gordon, 1970). Experiments involving gland ablation and replacement therapy have shed little light on the mechanisms except that for α_2AP globulin in the rat it has been possible to demonstrate an absolute requirement for

Corticosterone (Weimer and Benjamin, 1966; Weimer and Cogshall, 1967). Koj and Allison (1969) suggested that lysosomal disruption by injury might be the trigger for the acute phase reaction, but intravenous injection of lysosomal enzymes produced no change in plasma protein concentrations and there was no clear relationship between the events of the acute phase reaction after irradiation injury or turpentine injection in the rat and blood levels of various lysosomal enzymes (Koj, 1970).

Various factors are known to modify the acute phase reaction. Rat liver perfusion experiments emphasize the importance of good nutrition and the presence of cortisol and insulin for maximal production of protein (John and Miller, 1969; Miller and John, 1970) and studies in 6 strains of rats indicated that genetic differences were important (Weimer et al, 1972). The previous history of the animal was also important for a second stimulus will usually bring about a greater increase than the first and a third produced a different pattern of response (Weimer and Humelbaugh, 1967).

The importance, if any, of the acute phase reaction to the individual is not known but the localization of acute phase proteins in damaged tissue (Agostini et al, 1972) and experimental granulomata (Menninger et al, 1970; Hayle et al, 1971) has led to the suggestion that the proteins may be important in the repair process possibly by supplying a source of amino acids for the synthesis of new protein.

The pattern of the changes in the groups of patients with liver disease - a fall in the concentration of prealbumin and transferrin and a rise in easily precipitable glycoprotein, α_1-antitrypsin and caeruloplasmin is similar to the acute phase reaction already described. The most striking differences are the rise in α_2-macroglobulin in all groups with chronic liver disease, the absence of a large increase in haptoglobin, and a fall in some groups of serum concentrations of haemopexin. These findings suggest that the basic disturbance may indeed be the non-specific acute

phase reaction but that its full expression which depends on the presence of normal liver function, is modified by the limitations to synthesis imposed by the underlying liver disease which evoked the acute phase reaction. This suggestion is supported by the serial readings in Case 4 with active chronic hepatitis. Initially the concentrations of most proteins were reduced and it was only when liver function had improved substantially that hepatic synthesis was presumably adequate to increase the synthesis of the plasma proteins which take part in the acute phase reaction.

The changes in the individual proteins studied in the different disease groups will be discussed separately together with the relevant available information on the mechanisms controlling their plasma concentrations.

Prealbumin

is mainly if not wholly produced by the liver (Gitlin and Biasucci, 1969; Kaighn and Prince, 1971) and its turnover seems to be relatively fast. Studies with radioactive labelled protein showed the biological half life lay between 1.9 and 2.7 days (Oppenheimer et al, 1965; Socolow et al, 1965).

The factors controlling turnover are not fully understood but the higher levels in men than women, confirmed in the present study, suggests that hormonal influences may be important. Serum concentrations are increased by the progestogen ethinyl nortestosterone (Florsheim and Faircloth, 1964), norethandrolone (Braverman et al, 1971) and large doses of prednisone (Oppenheimer and Werner, 1966). Levels fall in pregnancy (Stabilini et al, 1968) and in common with albumin and transferrin in protein-calorie malnutrition (Ingenbleek et al, 1972). The rapid and invariable fall in concentration in the acute phase reaction discussed already is probably due to reduced synthesis although this is inferred from studies of catabolism of the protein (Oppenheimer et al, 1965; Socolow et al, 1965) and in some

patients the fractional catabolic rate may be increased.

Studies in patients with liver disease are unanimous that the plasma protein concentration is reduced. This was also our experience in hepatitis (Muller and Muller von Voigt, 1967; Smith and Goodman, 1971; Kindmark and Laurell, 1972), and in primary biliary cirrhosis (Weeke, 1973). The same is true in alcoholic cirrhosis (Marasini et al, 1968; Hallen and Laurell, 1972; Weeke, 1973) cryptogenic cirrhosis and active chronic hepatitis (Smith and Goodman, 1971; Hallen and Laurell, 1972). There are no kinetic data available for prealbumin in liver disease but the close correlation between prealbumin and albumin, haemopexin and other proteins whose concentration also fell suggests synthetic failure may be the cause.

The functional implication of the profound fall in prealbumin in liver disease is not entirely clear but the prealbumin thyroxine binding capacity must fall with a consequent redistribution of thyroxine onto thyroid binding globulin and albumin (Oppenheimer, 1968). Depending on the levels of thyroxine binding globulin a rise in the percentage plasma free thyroxine may occur (Hollander et al, 1967). However the absolute value of plasma thyroxine and the absolute thyroxine uptake into the tissues in liver disease are usually normal (Inada and Sterling, 1967). Low plasma levels of vitamin A have been recognised in liver disease for many years (Popper et al, 1943) and this is obviously due to the low levels of the binding proteins in the blood. However tissue levels are seldom much reduced unless there is malabsorption of the vitamin due to biliary obstruction and clinical deficiency is rare (Wright and Wright, 1971).

<u>α_1-antitrypsin</u>

The liver is the major if not the only site of α_1-antitrypsin synthesis (Wada et al, 1970; Kaighh and Prince, 1971) and the most striking evidence comes from human liver transplantation which restored blood levels to normal

in a homozygous deficient subject (Sharp, 1971). We also noted a change in α_1-antitrypsin phenotype in one of our transplant recipients.

The greatly raised plasma levels of α_1-antitrypsin in the patients with primary biliary cirrhosis agrees with the findings of Weeke (1973). Raised levels were also found in acute hepatitis by Muller and Muller von Voigt (1967) and Kindmark and Laurell (1972). No data was found in the literature on α_1-antitrypsin concentrations in patients with extrahepatic biliary obstruction but in view of the wide range of stimuli which increases plasma α_1-antitrypsin levels this finding is perhaps not unexpected. In the second series of patients with haemochromatosis, in the great majority of whom liver function tests were entirely normal, α_1-antitrypsin concentrations were normal. Review of the large plates from the series of patients with cryptogenic and alcoholic cirrhosis and active chronic hepatitis showed that the levels were normal or high in all patients although accurate quantitation was impossible because the protein had run off the plate in many cases. However high levels were found in the patients with active chronic hepatitis who were studied serially on miniplates and reports in the literature indicate that raised levels of α_1-antitrypsin are a common finding in patients with alcoholic cirrhosis (Marasini et al, 1972; Weeke, 1973; Hallen and Laurell, 1972), in cryptogenic cirrhosis (Hallen and Laurell, 1972), groups of patients with poorly characterized or a mixture of types of cirrhosis (Muller and Muller von Voigt, 1967; Brezin et al, 1971; Weeke, 1973) and chronic hepatitis (Muller and Muller von Voigt, 1967). We found no case of homozygous α_1-antitrypsin deficiency in screening our adult patients who presented with various forms of liver disease. The finding of one patient with heterozygous deficiency is fewer than expected from the known gene prevalence in the community and other cases probably passed undetected by the present technique as the serum concentration may

have been raised to normal by the stimulus to synthesis which liver disease seems to represent.

The stimulus for the increased plasma concentration in liver disease is unknown but it seems to occur in those groups of patients with the greatest disturbance in liver function. Similar increases are found due to a wide variety of non-specific stimuli as part of the acute phase reaction as has already been discussed.

The association of liver disease with α_1-antitrypsin deficiency was first reported by Ganrot et al, (1967b) who found two cases of cirrhosis and one primary hepatoma in a series of 50 adult patients with homozygous deficiency. None of these three cases had clinical pulmonary disease. No such cases were found in the present series and it appears that clinical liver disease in adults is rarely associated with α_1-antitrypsin deficiency. Sharp et al, (1969) described 10 children in 6 families who had cirrhosis in association with severe α_1-antitrypsin deficiency and examination of their sera later confirmed Pi ZZ patterns (Aagenaes et al, 1972). Since then there have been several further reports of the association of liver disease in children with severe α_1-antitrypsin deficiency (Johnson and Alper, 1970; Sharp, 1970; Aagenaes et al, 1972; Porter et al, 1972). Most of these children were jaundiced in the first few weeks of life with either a hepatitic or cholestatic biochemical picture and then, although liver function improved, slowly progressed to cirrhosis and portal hypertension. Two older children reported by Glasgow et al (1971) also developed emphysema. Several adults with emphysema as their presenting complaint have also been found to have cirrhosis (Pedersen et al, 1969; Berg and Eriksson, 1972; Cohen et al, 1973) but in most adult cases with emphysema associated with α_1-antitrypsin deficiency there is no clinical evidence of liver disease although like one of the cases in the present series liver function tests may be slightly disturbed (Aagenaes et al, 1972; De Lellis

et al, 1972). However, histological examination of the liver often shows focal fatty change, mild portal fibrosis and bile duct proliferation (Gherardi, 1971; Lieberman et al, 1972; De Lellis et al, 1972; Gordon et al, 1972) or true cirrhosis. In 14 homozygous deficient adults, of whom at least 10 had proven emphysema, severe fibrosis or true cirrhosis was found on liver biopsy in 8, and all of them were over 50 years old. It is of great interest that of these 8 no less than three had primary liver tumours (Berg and Eriksson, 1972). In the present cases it was not possible to obtain liver biopsy material.

Sharp et al, (1971) first demonstrated amorphous PAS positive staining globules in the hepatocytes of children with cirrhosis associated with α_1-antitrypsin deficiency and by specific fluorescent tagged antibody staining showed that these were deposits of α_1-antitrypsin. These findings have been confirmed in homozygous adults with emphysema and minimal liver disease and also in adults with heterozygous (Pi^{MZ}, Pi^{SZ}, and Pi^{SS}) deficiency (Lieberman et al, 1972; De Lellis et al, 1972, Gordon et al, 1972; Aagenaes et al, 1972). The amount of α_1-antitrypsin demonstrable in the liver cells was much greater in the homozygous deficient subjects but there was no correlation between the amount of α_1-antitrypsin in the liver and the degree of hepatic damage. Electron microscopy of these livers showed masses of electron dense material lying with the rough endoplasmic reticulum. These appearances suggest that the primary abnormality may be an inability of the hepatocyte to release the protein from the cell, but whether this is related to changes in the carbohydrate moiety or to alterations in the amino acid sequence must await further investigation. Recent work on the release of rat albumin from the liver cell suggests that a larger precursor molecule is split before albumin appears in the plasma (Geller et al, 1972) and failure of such a releasing mechanism might apply to α_1-antitrypsin deficiency. Attempts to increase plasma levels by treatment with

phenobarbitone, corticosteroids (Porter et al, 1972) oestrogens and typhoid vaccine have been unsuccessful (Sharp, 1971; Lieberman et al, 1972). The plasma survival of transfused radio-active labelled α_1-antitrypsin in these patients is normal but the biological half-time of 4-6 days is too short to make this a practical form of therapy (Kueppers and Fallat, 1969; Makino and Reed, 1970). The plasma half-life of the abnormal α_1-antitrypsin phenotypes has not been determined.

Detailed evaluation of other liver produced plasma proteins in the present series of 7 homozygous adult subjects showed no significant changes except that α_2-macroglobulin was greatly raised in 2 cases. This was also the experience of Ganrot et al, (1967b) in a series of 50 homozygous deficient (Pi^{ZZ}) patients in whom the mean α_2-macroglobulin concentration was 135% of the value in the standard serum. There was no difference between the levels in patients with advanced pulmonary disease and those who were clinically well and one cannot postulate that the $_1$-antitrypsin activity of high concentrations of α_2-macroglobulin (Ganrot, 1967) is exerting a protective effect. In the present cases the highest serum α_2-macroglobulin level occurred in a patient severely disabled by emphysema. It is not known whether the raised levels of α_2-macroglobulin were a reflection of hepatic fibrosis or cirrhosis.

The clinical presentation of patients with homozygous deficiency of α_1-antitrypsin ranges from hepatitis in neonates which often progresses to cirrhosis, to pulmonary emphysema or occasionally cirrhosis in adults. Some patients remain asymptomatic throughout their lives but sub-clinical changes in both lungs and liver are probably common. Patients with both pulmonary and hepatic symptoms occur both in childhood and adulthood but are rare as are families with both types of disease (Sharp et al, 1969). The factors governing the clinical expression of α_1-antitrypsin deficiency are unknown but smoking seems to be important in the pathogenesis of emphysema (Hutchison et al, 1972). Porter et al (1972) found Australia

antigen in the serum of 3 of 5 children presenting with hapatitis and suggested this might be an important trigger factor but other groups have failed to substantiate this in larger series (Sharp, 1971; Aagenaes et al, 1972). It would be of great interest to know whether the clinical outcome of viral hepatitis, drug or alcohol induced liver injury was different in adult homozygous deficient patients from control subjects with normal or raised levels of α_1-antitrypsin.

Group component

The liver was shown to be the site of formation of group component by Prunier et al (1964) who cultured human tissue biopsies in vitro with radioactive labelled amino acids and then performed immunoelectrophoresis of the supernatant followed by autoradiography of the plate. No other tissue tested synthesised group component and this conclusion is supported by the complete change in protein phenotype which accompanies transplantation of the liver (Kashiwagi et al, 1968).

It is therefore not surprising that low plasma concentrations have been reported in severe hepatic failure (Kitchin and Bearn, 1965; Cleve and Dencker, 1967) but in cirrhosis and acute and chronic hepatitis with less severe damage levels were normal (Cleve and Dencker, 1967). In the present studies the concentration of this protein was normal in all diagnostic groups studied. Minor increases have been noted in some patients with rheumatoid arthritis (Cleve and Dencker, 1967) and malignant disease (Hughes, 1971) but the concentration of group component changes little in response to inflammation and other stimuli provoking the 'acute phase reaction'.

α_2-Macroglobulin

was raised in all groups of patients with chronic liver disease in the present study but not in those with hepatitis or extrahepatic obstruction. The first report of raised levels in chronic liver disease

was in a group of patients with active chronic hepatitis (Zlotnick and Rodnan, 1962) and this finding has been confirmed many times in different types of cirrhosis (Cleve and Strohmeyer, 1967; Housley, 1968; Brezin et al, 1971; McSween et al, 1972; Hallen and Laurell, 1972; Weeke, 1973). However normal values were found by Marasini et al (1972) in 91 patients with alcoholic cirrhosis and by Muller and Muller von Voigt (1967) in a series of patients with poorly characterised chronic hepatitis and cirrhosis. The normal levels in our patients with hepatitis are in agreement with the findings of Cleve and Strohmeyer (1967), Muller and Muller von Voigt (1967) and Kindmark and Laurell (1972) who measured α_2-macroglobulin serially during the course of the illness in 23 patients. The concentration of this protein is not subject to large fluctuations like the acute phase reactants and serial readings in our patients with active chronic hepatitis showed little change although the concentration of other proteins altered strikingly.

Liver has been shown to synthesise α_2-macroglobulin (Hochwald et al, 1961; Prunier et al 1964). The maintenance of a high plasma concentration in the face of severe liver disease suggests the possibility of an extra-hepatic site of synthesis and this was shown by Prunier et al (1964) who studied the incorporation of radio-active amino acids by biopsies of human skeletal muscle. However the ability of α_2-macroglobulin to bind small molecules which may be synthesised by the tissue in question throws serious doubt on the significance of these results (Stecher and Thorbecke, 1967).

Turnover studies with iodinated α_2-macroglobulin indicated a biological half life of between 8 and 11 days (Kluthe et al, 1967) but as the protein was not pure this must be regarded as an approximation. A more recent attempt to obtain valid kinetic data shows similar results but again the preparation of the protein was unsatisfactory (Norberg et al, 1970). Little is known about the factors controlling its concentration in the

plasma. It does not take part in the acute phase reaction (see above). Although it was not affected by oestrogens in a series of men given high doses for carcinoma of the prostate (Adham et al, 1968), most series, including the present one indicate that plasma levels are slightly higher in women than men (James et al, 1966; Ganrot and Schersten, 1967; Muller et al, 1970) and levels rise by about 20% in pregnancy (Ganrot and Bjerre, 1967; Mendenhall, 1970) and in women taking the contraceptive pill (Laurell et al, 1967; Horne et al, 1970). Greatly elevated levels are common in renal disease with proteinuria but there is disagreement about whether there is a correlation with the degree of urinary protein loss (Hedfors et al, 1971; Horne et al, 1972). Increased plasma concentrations of α_2-macroglobulin are also found in patients with diabetes mellitus (Ganrot et al, 1967a; Muller et al, 1970) especially in association with longstanding disease and proteinuria.

The factors leading to the increased levels of α_2-macroglobulin in liver disease are unknown but it is a feature only of chronic disease. Whether this is related to the long time course or to the nature of the disease is unknown. The finding of raised α_2-macroglobulin levels in a patient with a prolonged abnormality of liver function tests following acute viral hepatitis would suggest the development of active chronic hepatitis and might be of diagnostic value. In chronic liver disease there is evidence for both increased intravascular coagulation and more rarely increased fibrinolysis (Roberts and Cederbaum, 1972) but whether the rise in α_2-macroglobulin concentration contributes to the inbalance because of the increased plasma binding capacity for both thrombin and plasmin in speculative. In a study of plasma proteins and the fibrinolytic system in patients with chronic glomerulo-nephritis (Wardle et al, 1970) no correlation was found between the diminished fibinolytic activity and the rise in α_2-macroglobulin.

Low plasma levels are uncommon but were seen in one patient dying of fulminant hepatic failure not included in the present series and have been reported after infusions of streptokinase which converts plasminogen to plasmin (Nilehn and Ganrot, 1967). The plasmin combines irreversibly with α_2-macroglobulin and the complex is removed from the circulation probably by the reticuloendothelial system as has been shown in experimental animals for the trypsin-α_2-macroglobulin complex (Ohlsson, 1971). Low levels of α_2-macroglobulin persist for up to 16 days which is in keeping with the relatively slow turnover of this protein. A drop in α_2-macroglobulin concentration has recently been reported following bone surgery in children and it was suggested that this was due to the formation of complexes of α_2-macroglobulin with proteases released into the circulation by the bone trauma (Dickson and Manning, 1972). Animal experiments suggest that severe reduction of α_2-macroglobulin levels may be dangerous for further release of proteases which cannot be bound and inactivated will result in massive fibrinolysis (Ohlsson et al, 1971). It is possible that this mechanism may account in part for excessive fibrinolytic activity which is found in some patients with acute hepatic failure (Gallus et al, 1972).

Caeruloplasmin

The liver seems to be the major source of serum caeruloplasmin. This is based on evidence from in vitro incorporation of radioactive labelled amino acids (Hochwald et al, 1961; Prunier et al, 1964), localisation by specific immunofluorescence and release of the protein from liver slices which was prevented by inhibitors of protein synthesis (Neifakh et al, 1969). Although copper can be removed and recombined with the protein in vitro (Scheinberg and Sternlieb, 1960), there is no evidence for exchange in vivo and the copper atoms are incorporated before release of the protein from the liver (Sternlieb et al, 1961; Holzman and Gaumnitz, 1970a). There is now

evidence using immunological techniques that a small proportion (10-20%) of caeruloplasmin in normal human serum is devoid of copper oxidase activity (Carrico et al, 1969). This apocaeruloplasmin has slight antigenic and electrophoretic differences from holocaeruloplasmin and conversion occurs slowly with storage of serum and can be produced by removing the copper in vitro with potassium cyanide. Abnormally high concentrations of plasma apocaeruloplasmin may occur in Wilson's disease (Carrico et al, 1969) and will be detected by immunological techniques but not by the oxidase method.

The biological half life of caeruloplasmin whether labelled with ^{131}I (Kekki et al, 1966), with ^{64}Cu (Sternlieb et al, 1961) or ^{67}Cu (Waldmann et al, 1967) was closely similar at between 4 and 6 days. Turnover studies with apocaeruloplasmin in the rat showed the half life was much shorter than the holoprotein (Holzman and Gaumnitz, 1970b) and Morell et al (1971) have also shown that removal of sialic acid residues considerably reduces the biological half life. Little is known about the sites of catabolism under normal circumstances but losses in bile (Aisen et al, 1964), the gastro-intestinal tract (Waldmann et al, 1967), and urine are minor.

Several factors are known to influence the concentration of plasma caeruloplasmin including various hormones. Oestrogen administration causes a striking rise (Russ and Raymunt, 1956) and this effect explains the increased plasma concentrations found in women on the oral contraceptive pill and during pregnancy (Laurell et al, 1967; Song et al, 1970) as progestogens alone have no effect (Laurell et al, 1969; Briggs et al, 1970). Removal of either the adrenals or the thyroid gland in rats results after 4 weeks in a sustained increase in caeruloplasmin levels which are restored to normal by hormone replacement (Evans et al, 1970a). Several studies in the developing countries have shown that caeruloplasmin levels fall early (like transferrin and prealbumin) in protein calorie malnutrition (Reiff

and Schnieden, 1959) but levels may be misleadingly normal if synthesis is stimulated by infection which is so often part of the clinical picture (Gopalan et al, 1963; Ismadi et al, 1971). The prominent part played by caeruloplasmin in the acute phase reaction has been mentioned already. Evidence that liver copper concentration might have a specific influence on caeruloplasmin synthesis came from the studies on endocrine gland ablation in which raised serum copper levels were found to accompany the rise in caeruloplasmin (Evans et al, 1970a). In a recent study using the incorporation of radioactive labelled lysine direct evidence for the induction of caeruloplasmin synthesis by copper has been presented (Evans et al, 1970b). This effect was blocked by inhibitors of protein synthesis including actinomycin D suggesting that copper was acting at the level of transcription. In a child with copper intoxication due to absorption of copper sulphate applied to burns a rise in caeruloplasmin concentration was noted and was tentatively attributed to the copper load (Holtzman et al, 1966). However the child had a general systemic upset with disturbance of liver function and a haemolytic anaemia and as no other specific proteins were measured it is impossible to rule out an acute phase response.

Serum caeruloplasmin levels were significantly raised in all groups of patients in the present study except those with cryptogenic cirrhosis and primary haemochromatosis. The most strikingrrise was seen in primary biliary cirrhosis and extrahepatic obstructive jaundice and it is interesting that liver copper content is usually greatly raised in both these conditions (Smallwood et al, 1968). Very high levels of plasma caeruloplasmin have been noted before in obstructive jaundice from whatever cause (Bearn and Kunkel, 1954; Pineda et al, 1962; Hauftova and Slavicek, 1963, Ventura et al, 1967; Gault et al, 1966; Prellwitz et al, 1969) and also in active chronic hepatitis (Bearn and Kunkel, 1954). The mean level in patients with alcoholic cirrhosis was significantly raised although there was a wide

scatter of readings and similar results are reported by other workers (Gubler et al, 1957; Ventura et al, 1967) whereas Hauftova and Slavicek (1963) found normal or low values in 19 alcoholic patients. Many alcoholics are malhourished and this may have influenced the findings. The normal or only slightly elevated levels in the patients with hepatitis and cryptogenic cirrhosis agree with the results of other workers (Pineda et al, 1962; Hauftova and Slavicek, 1963; Cault et al, 1966; Trip et al, 1969). In severe acute or chronic liver disease with parenchymal cell failure, low levels of caeruloplasmin may be found and this may then cause diagnostic confusion with Wilson's disease (Walshe and Briggs, 1962; Gault et al, 1966). However levels may rise to normal if liver function improves.

Caeruloplasmin can be split into two fractions on a hydroxyapatite column, type I constituting about 86% in normal people (Trip et al, 1969). Fractionation of sera with differing total concentrations of plasma caeruloplasmin from patients with liver disease showed that the proportion of type I was reduced and it was suggested that only this fraction was synthesised in the liver. This observation requires further study.

Haptoglobin

The liver has been clearly shown to be the major site of haptoglobin synthesis by a variety of methods including incorporation of radioactive leucine and galactose by the isolated perfused rat liver (Krauss and Sarcione, 1964) and liver slices in vitro (Prunier et al, 1964; Wada et al, 1970a). Using immunoelectrophoresis of the incubation fluid followed by autoradiography to study the labelled proteins the latter group showed that human spleen and rat spleen and lymph nodes also appeared to synthesise haptoglobin although the quantities are probably small. No haptoglobin synthesis could be demonstrated by Alper et al (1965) in the hepatectomised dog and following transplantation in man the haptoglobin type was shown to change to that of the donor (Merrill et al, 1964; Kashiwagi et al, 1968). No

evidence for persistence of the patients previous haptoglobin type was found in follow up studies over 64 days (Kashiwagi et al, 1968). A study with fluorescent anti-haptoglobin antibody demonstrated fluorescence largely in the parenchymal cells although some was also present in the Kupffer cells (Peters and Alper, 1966). Immunofluorescence is, of course, no proof that that cell is synthesising the protein.

The mean biological half life of the protein labelled with radioactive iodine in two series of normal subjects was 4.5 days and 3.7 days (Moretti et al, 1963; Bottiger and Molin, 1968). This agrees closely with data derived by Noyes and Garby (1967) from the rate of return of haptoglobin plasma levels to normal after an injection of free haemoglobin and the reduction in specific activity of haptoglobin after in vivo pulse labelling with ^{14}C-glucosamine or ^{35}S-methionine (Dobryszycka et al, 1969). Freeman (1964) showed in a series of anaemic patients, some of whom had shortened red cell survival and markedly reduced plasma haptoglobin concentration, that although the fractional catabolic rate (the percentage of the intravascular pool catabolised/day) varied greatly the absolute catabolic rate (the weight of the protein catabolised daily) did not vary significantly. Hence the liver did not increase haptoglobin synthesis in response to the low plasma concentration. On the other hand the absolute and fractional turnover was greatly increased in the series of patients with raised haptoglobin levels studied by Dobryszycka et al (1969) after burns and other trauma.

The life span of the normal red blood cell is about 120 days and this means that 6-7g. of haemoglobin are released for catabolism daily. When this is compared with the weight of haptoglobin catabolised per day (about 1.5g/day) and the total plasma haemoglobin (about 0.3mg/100ml) it is obvious that the majority of haemoglobin breakdown must be extravascular and the major site is thought to be the reticulo-endothelial system (Bunn, 1972).

The haptoglobin-haemoglobin complex disappears rapidly from the circulation with a biological half time of 10-30 minutes (Garby and Noyes, 1959; Freeman, 1964). Experiments in animals injected with radioactive labelled haemoglobin have shown that the liver is a major site of uptake with lesser amounts in the spleen and bone marrow (Keene and Jandl, 1965). Because of the distribution of radioactivity and by analogy with the site of removal of damaged red blood cells it has been widely assumed that the haptoglobin-haemoglobin complex is taken up by the reticuloendothelial system (Muller-Eberhard, 1970; Bunn, 1972). However histological proof was not provided and blockading the reticuloendothelial system with colloidal carbon did not alter the kinetics of clearance (Franklin et al, 1960). In the autoradiographic study of rat liver after injection of radioactive labelled haptoglobin-haemoglobin complexes reported by Wada et al (1970b) radioactivity was detected mainly in the Kupffer cells but the dose of protein injected was unphysiological and the finding of large amounts of radioactivity in the renal tubules suggests that the preparation may have been unsatisfactory. On the other hand Goldfischer et al (1970) demonstrated haemoglobin in the hepatic parenchymal cells by a histochemical technique following injection of varying doses of unlabelled free haemoglobin. Two more recent and critical studies have demonstrated clearly that the great majority of haemoglobin whether free or complexed with haptoglobin is removed by the parenchymal cells. Bissell et al (1972) identified the hepatic cell types by enzymic disruption of the liver and fractionation into pure isolates of parenchymal cells and sinusoidal cells. After injection of radioactive labelled haemoglobin 85-95% of the radioactivity was associated with the parenchymal cells and levels of microsomal haem oxygenase, the first enzyme in the chain responsible for degradation of haemoglobin to bilirubin, were greatly increased. The other study by Finch's group (Cook et al, 1972) confirmed these results using a different technique.

The liver therefore plays a central role in the synthesis and catabolism of haptoglobin. Plasma levels rapidly increase as part of the acute phase reaction and following treatment with cortisone and anabolic steroids (Krauss, 1968; Barbosa et al, 1971). On the other hand oestrogens and pregnancy result in a fall in plasma concentration (Laurell et al, 1967; Song et al, 1970) but progestogens alone have no effect on plasma levels (Laurell et al, 1969).

In diseases of the liver either reduced or increased plasma concentrations of haptoglobin may be found and this was originally suggested as the basis of a diagnostic test (Owen et al, 1961). However interpretation of the individual result is difficult because of the wide scatter of values both in patients and normal subjects. Levels were not significantly different from normal in the present series of haemochromatotic subjects and this agrees with the findings of Williams et al (1961) and Bock et al (1969). Both these groups and the present study and also Zlotnick and Rodnan (1962) found much reduced levels in active chronic hepatitis and in several of our patients there was no visible precipitation arc. There is disagreement in the literature about the findings in acute viral hepatitis and this probably stems from the variable times in the course of the illness that samples have been taken. Serial readings show that levels are normal or high in the first week or two but they tend to fall below normal as the serum bilirubin rises to a peak (Owen et al, 1961; Williams et al, 1961; Kindmark and Laurell, 1972). The mean in the present series was normal although in 4 samples the concentration was low. Other series in which only one sample has been studied have given normal (Nyman, 1959; Muller and Muller von Voigt, 1967; Prellwitz et al, 1969) or low mean values (Kallai et al, 1966). Nyman (1959) noted that in infectious mononucleosis with hepatitis the levels were consistently low possibly due to the haemolysis which sometimes occurs in that disease. In alcoholic cirrhosis low

levels were found although the mean did not differ significantly from normal and low values have also been reported by Nyman (1959), Weeke (1973) and Marsini et al (1972). In none of these cases was there frank haemolytic anaemia. There are reports of low levels in non alcoholic cirrhosis (Nyman, 1959; Williams et al, 1961; Kallai et al, 1966; Prellwitz et al, 1969; Hallen and Laurell, 1969; Brezin et al, 1971) but the exact type of disease is not clearly identified in most reports. Some cases would probably have been classified as active chronic hepatitis in the present study and low values are common in that disease.

In primary biliary cirrhosis levels were normal in the present series in agreement with Williams et al (1961) and Weeke (1973) but in extra-hepatic obstructive jaundice the mean level was raised although there was a wide scatter of individual results. Raised levels in about half the cases of extrahepatic obstructive jaundice have been reported by several authors (Nyman, 1959; Owen et al, 1961; Kallai et al, 1966; Prellwitz et al, 1969) but as raised levels are found with both calculous and malignant obstruction the finding is of no help in differential diagnosis.

The mechanism of these changes in liver disease is uncertain. Most authors have failed to find a significant correlation with any of the liver function tests although Owen et al (1959) found some correlation between haptoglobin and serum albumin suggesting that the low levels were related to impaired hepatic synthesis. No turnover studies with radioactive labelled protein have been made in liver disease to settle this point. There does, however, seem to be some relation to disease activity and serial levels in patients with active chronic hepatitis showed that haptoglobin levels rose with improvement in liver function tests and a similar pattern has been shown in acute viral hepatitis. An alternative explanation for the low levels is that the catabolic rate of haptoglobin is increased by shortened red cell survival. However, Williams et al,

(1961) were unable to demonstrate any correlation between the red cell survival and haptoglobin level. In some patients with obstructive jaundice the plasma haptoglobin level was high although red cell life was severely shortened. Obviously the plasma level is the resultant of synthesis and catabolism and both may be altered to a variable degree. The present results support Nyman's (1959) comment that a high haptoglobin concentration argues for obstructive jaundice while the normal or low concentration is ofllittle diagnostic value.

Haemopexin

The human foetal liver (Gitlin and Biasucci, 1969) and the isolated perfused rat liver (M. Rothschild, personal communication) have been shown to produce haemopexin and no other tissue was shown to incorporate radioactive amino acids into haemopexin in vitro (Thorbecke et al, 1973). The factors controlling synthesis are largely unknown. A slight rise has been reported in pregnancy (Ganrot, 1972) but the effects of oestrogens alone are unknown. Although the concentration may increase slightly as part of the 'acute phase reaction' (see above) this is seldom a striking finding (Kushner et al, 1972).

Low values are regularly found in haemolytic states in which there is severe depletion of haptoglobin (Sears, 1968; Hanstein and Muller-Eberhard, 1968; Muller-Eberhard et al, 1968; Braun and Ali, 1971; Eyster et al, 1972) and may occur in haemorrhagic pancreatitis without a fall in haptoglobin levels due to release of free haem into the circulation (Sears, 1968). This unusual finding of depletion of haemopexin with normal levels of haptoglobin has also been described in porphyria cutanea tarda possibly due to combination of haemopexin with the raised blood porphyrins (Meiers and Ippen 1968). Raised levels are described in diabetes mellitus (Cleve et al, 1968) myelomatosis (Rentsch 1969), and Hodgkin's Disease (Korinek,

1969), Crohn's disease (Weeke and Jarnum, 1971) and active tuberculosis (Clarke et al, 1970a).

The raised levels in the first series of patients with haemochromatosis have been discussed previously and it seems likely that this was due to a technical error. Normal levels in close relatives and the patients with secondary iron overload are against there being either a genetic disturbance in haemopexin metabolism or a relationship with excess body iron stores.

In general low levels of haemopexin were found in those liver diseases with the most marked disturbance in liver function. The low levels in acute viral hepatitis are in agreement with other authors (Muller and Muller von Voigt, 1967; Braun and Aly, 1971; Kindmark and Laurell, 1972) and low levels were also found in small series of patients with extrahepatic obstruction (Braun and Aly 1970, 1971) and primary biliary cirrhosis (Weeke, 1973). In alcoholic and cryptogenic cirrhosis both normal and reduced levels are reported possibly reflecting the degree of decompensation of liver function in the different series (Muller and Muller von Voigt, 1967; Braun and Aly, 1970; 1971; Brezen et al, 1971; Hallen and Laurell, 1972; Weeke, 1973). The cause of the fall in haemopexin concentrations in liver disease is not clear but reduced synthesis seems more likely than consumption due to haemolysis as the haptoglobin levels, except on rare occasions, were not depleted and the fall in haemopexin was significantly correlated with reduction in the levels of other liver produced proteins. The point can only be settled by turnover studies with radioactive-labelled haemopexin.

Haemopexin and other plasma proteins in hepatic enzyme induction

Many foreign compounds such as drugs and insecticides stimulate the activity of enzymes in the endoplasmic reticulum of the liver, an effect known as enzyme induction (Conney, 1967; Kuntzman, 1969). Strictly interpreted the term "enzyme induction" implies a selective increase of an

enzyme relative to the concentration of microsomal protein in the cell. Not all microsomal enzyme systems are stimulated but particularly the microsomal mixed function oxidase system. This consists of $NADPH_2$, cytochrome P_{450} and a linking electron transport system consisting of cytochrome P_{450} reductase and NADPH cytochrome C reductase (Parke, 1971). The system has diverse activities and is particularly concerned with the oxidation of drugs and hormones, biosynthesis of cholesterol, oxidation of fatty acids and prostaglandins. Many drugs and chemicals are known to stimulate the system and they have in common the property of being fat soluble and the tendency to localise in the endoplasmic reticulum. At least two kinds of inductive process can be distinguished - one by phenobarbitone and most other drugs which act as inducers and the other by the polycyclic hydrocarbons. Phenobarbitone-like inducers cause increases in NADPH cytochrome C reductase and cytochrome P_{450} and this increases the amount of the cytochrome P_{450}-substrate complex and its rate of reduction. Polycyclic hydrocarbons such as 3-methylcholanthrene (3-M.C.) induce the formation of a variant of cytochrome P_{450} which has different affinities for the various drug substrates than does the normal form. This kind of inducer does not increase either NADPH cytochrome C reductase or the rate of cytochrome P_{450} reduction (Gillette, 1971).

The mechanism of enzyme induction is not known but it almost certainly involves the synthesis of new protein as incorporation of radioactive amino acids into microsomal protein has been shown in vivo and in vitro, and the induction process can be inhibited by puromycin and actinomycin D (Parke, 1971). Most inducers have also been shown to increase the level of hepatic Δ amino laevulinic acid synthetase (ALA synthetase) (Tschudy and Bonkowsky, 1972) which is the rate limiting enzyme in the biosynthesis of haem (Granick, 1966) and this increase in haem maybe required for the increased synthesis

of the haem containing enzymes cytochrome P_{450} and cytochrome b_5. This concept is supported by the fact that when haem synthesis is partly inhibited by 3 amino 1,2,4-triazole (aminotriazole) induction of cytochrome P_{450} by phenobarbitone and 3,4-benzpyrene is inhibited (Baron and Tephley, 1969a, 1969b). Triazole inhibits ALA dehydratase, the second enzyme in the haem synthetic pathway, and has no effect on protein synthesis in general as is shown by the finding that phenobarbitone increased the levels of NADPH cytochrome C reductase in the usual manner. The sequence of events in the induction of microsomal enzymes therefore seems to be induction of ALA synthetase, increased incorporation of glycine into microsomal haem and then increased activity of cytochrome P_{450} and other oxidases (Tephly et al, 1971). However not all haem formed is incorporated into haem containing enzymes and there is evidence for a hepatic free haem pool (Garner and McLean, 1969; Druyan and Kelly, 1972). The control of ALA synthetase has attracted much attention and there is good evidence that haem, the end product of the pathway controlled by ALA synthetase, controls this enzyme by an induction-repression mechanism (Granick, 1966; Tschudy and Bonkowsky, 1972), although as haem also inhibits the enzyme directly in vitro there is also the possibility in some circumstances of feedback inhibition.

3-M.C. allylisopropylacetamide (AIA) and phenobarbitone, all of which are potent enzyme inducers, have been shown also to induce the synthesis of haemopexin in rabbits (Ross and Muller-Eberhard, 1970a) resulting in a twofold rise in the concentration of this protein in the blood. That this effect is due to de novo protein synthesis was shown by the inhibitory effects of ethionine (Ross and Muller-Eberhard, 1970a, b) and cyclohexamide (Smibert et al, 1972). It was established that the induction of the protein was not dependant on the simultaneous synthesis of haem by studying the effects of aminotriazole and lead acetate and 3,5-dicarbethoxy-1,4-dihydro-collidine (DDC) which inhibit ALA dehydratase and ferrochetalase

(Goldberg, 1972). Haemopexin whose role in haem catabolism has been discussed has been shown in vitro to prevent the inhibitory effect of high haem concentrations on ALA synthetase (Warnick and Burnham, 1971) and to remove haem in vitro from the microbial enzyme cytochrome P_{450} resulting in a reduction in activity of this enzyme (Muller-Eberhard et al, 1969b). It has been suggested therefore that haemopexin may serve to regulate the drug metabolising enzymes by binding excess haem produced by ALA synthetase induction. This surplus haem, if haemopexin were not present to bind it, would otherwise inhibit the metabolism of drugs and hormones resulting in the accumulation of toxic substances (Smibert et al, 1972).

There was no evidence of induction of haemopexin synthesis reflected by increased plasma levels either in the two normal subjects taking phetharbital, a non-hypnotic barbiturate, nor in the induced epileptics. However, a minor increase might not have been reflected in a detectable increase in plasma levels because of the distribution of the protein in the extravascular space as well as the plasma compartment (Lane et al, 1972). Equally an increase in synthesis may have been balanced by increased catabolism. An answer to this problem must await turnover studies with radioactive labelled protein. The evidence that hepatic enzyme induction had been achieved in the two groups of subjects rests on the fall in plasma bilirubin in the two normal subjects (Thompson et al, 1969) and the raised urinary D-glucaric acid excretion in both groups (Hunter et al, 1971a). Evidence for hepatic enzyme induction based on these grounds has been given before both for normal subjects and patients treated with phetharbital (Hunter et al, 1971c) and epileptics on long-term anti-convulsant therapy (Hunter, 1971b). Bucolome has been reported to reduce the plasma bilirubin levels in patients with Gilbert's disease (Yamamoto and Sakamoto, 1971; Yamamoto et al, 1972) and studies in rats showed increased activity of

para-nitrophenol glucuronyl transferase and proliferation of the smooth endoplasmic reticulum. It was not possible, however, to show increased bilirubin glucuronide formation (Yamamoto and Sakamoto, 1971). We found no evidence of hepatic enzyme induction by bucolome with the dosage schedule used.

It is now known that treatment with phenobarbitone and other enzyme inducers has far reaching and varied metabolic effects (Conney, 1967; Kuntzman, 1969) not only in the liver but also in other tissues. For instance phenobarbitone administration in the rat has been shown to be followed by an increase in the smooth endoplasmic reticulum in the small bowel (Thomas et al, 1972a) and increased activity of several enzyme systems including those incorporating acetate into cholesterol (Middleton and Isselbacher, 1969). It has also been shown to increase iron absorption in the rat possibly by increasing the synthesis of a carrier protein (Thomas et al, 1972b). The hepatic microsomal metabolism of cortisol and sex hormones is increased (Parke, 1971).

Adverse clinical consequences of hepatic enzyme induction are being increasingly recognised. These include osteomalacia in epileptics on long-term anti-convulsant drugs (Hunter et al, 1971b; Sotanienu et al, 1972) thought to be due to accelerated catabolism of cholecalciferol (vitamin D) and 25-hydroxycholecalciferol leading to diminished circulating and tissue levels of biologically active vitamin D metabolites (Hahn et al, 1972). It was of interest that these authors found the rise in serum alkaline phosphatase was due to the liver isoenzyme in the first few months of treatment. The serum alkaline phosphatase was raised in 6 of the 15 patients in the present series but the isoenzyme pattern was not determined. No patient had hypocalcaemia but the series is small and the prevalence of hypocalcaemia in adult epileptics on long-term anti-convulsant

therapy may be as low as 19% (Hahn et al, 1972). It is probably related to the dietary vitamin D intake. No attempt was made to assess any radiological changes in the skeleton in the present patients.

No systemic study of the effects of hepatic enzyme induction on the concentration of a range of plasma proteins has been published but Ross and Muller-Eberhard,(1970a) found no change in the concentration of transferrin at a time when the haemopexin levels were greatly elevated. In the present study on the two normal subjects no protein was found to alter significantly but the course of treatment may have been too short. In the epileptics, all of whom had been on anti-convulsant therapy for more than a year, the raised urinary D-glucaric acid excretion is consistent with hepatic enzyme induction in 13 of the subjects and in agreement with Hunter (1971a) we found a correlation between the dosage of anti-convulsant taken and the amount of glucaric acid excreted. The 5 proteins which were present at highly significantly increased concentration form an unusual grouping quite unlike that found in the acute phase reactions. The mechanism and significance of the change is quite unknown. In a similar study of 9 epileptics (Clarke et al, 1970c) mean levels of α_1-antitrypsin, caeruloplasmin and group component were raised but only the latter reached statistical significance. Details of drug treatment were not given. Raised caeruloplasmin levels in epilepsy were also noted by Abood et al (1957) and Vuyze and Visser (1966). Grob and Herold (1972) have reported low levels of IgA and β_1AC globulin in a group of patients taking hydantoins and Cantu and Schwab (1966) found the caeruloplasmin levels were higher in 17 patients on phenytoin than in the controls. Serial readings in 5 patients showed the levels had risen significantly after two weeks treatment and Brunia and Buyze (1972) have recently reported a significant elevation in serum copper in epileptics being treated with hydantoin but not those taking

ethosuxamide. A variety of plasma protein disturbances have also been reported in functional psycoses such as schizophrenia (Fessell, 1961; Seal and Eist, 1966) but the relationship, if any, of these observations to hepatic enzyme induction is uncertain. There is need for a systematic survey correlating the concentration of various plasma proteins with other metabolic parameters including glucaric acid excretion in a larger group of patients receiving long-term anti-convulsant treatment.

Transferrin

The liver is the major site of transferrin synthesis but lymphoid tissue is also active. Several groups of workers have shown incorporation of radioactive labelled amino acids into transferrin by liver and lymphoid tissue in vitro using both human and animal tissue (Hochwald et al, 1961; Prunier et al, 1964; Stecher and Thorbecke, 1967; Morgan, 1969; Soltys and Brody, 1970). The ability of lymphoid tissue to make transferrin has been confirmed many times perhaps most strikingly by Phillips and Thorbecke, (1966) who found that chimeras produced by injecting rat marrow into irradiated mice developed rat transferrin in the circulation. The liver of these animals produced only mouse transferrin. Lane (1968) has demonstrated transferrin in the liver by immunofluorescence and the increase in the degree and extent of staining which follows acute blood loss was taken as evidence in favour of hepatic synthesis. Using a perfused isolated calf liver Goldsworthy et al (1970) showed incorporation of radioactive labelled amino acids into transferrin and calculated that the liver was probably the major site of production in vivo. This conclusion is in agreement with the results of Morgan (1969) studying the synthetic capacity of rat tissues in vitro.

The intracellular events in transferrin synthesis by hepatocytes have recently been studied by Morgan and Peters (1971b). Using the incorporation of ^{14}C-leucine they showed that molecules of transferrin were complete in

2 minutes but that they remained in the microsomal fraction till their release from the cell started at 30 minutes. As the mean time of release after giving the label was at 80 minutes the rate of transit through the cell is substantially slower than for albumin where the mean transit time is 23 minutes.

The factors controlling transferrin synthesis are still incompletely understood and are confused by apparent differences between species of experimental animal and in the older literature by the measurement of total iron-binding capacity rather than transferrin. There maybe quite major differences between these measurements as will be discussed later. There are two main theories to account for the facts known about the regulation of transferrin synthesis, either that it is related to the plasma iron or body iron stores or that it is influenced by the balance in the body between oxygen supply and the oxygen demand of the tissues. An older view that the influence of erythropoetic activity is important has not been supported by more recent work (Morgan, 1962; Awai and Brown, 1963).

Weinfeld (1964) presented evidence that there was a correlation between iron stores in the liver and marrow (measured directly in biopsy specimens) and total iron-binding capacity (TIBC) of the plasma. In keeping with this relationship was the finding that the TIBC was higher than predicted from the curve in non-anaemic blood donors and patients who while not anaemic had had a recent haemorrhage. He also explained the lowered TIBC found in the anaemia of infection as being due to the increased iron stores which are found. In keeping with this hypothesis is the depression of the TIBC found in iron overload with or without secondary haemochromatosis (Bothwell and Finch, 1962; Barry et al, 1968; Johnson, 1968). However, Morgan (1966; 1969) and Tavill and Kershenobich (1972) were unable to show any change in transferrin synthesis in the rat following iron overload although the latter workers did show that in nutritional iron deficiency intravenous iron

reduced transferrin synthesis without altering the haemoglobin level. From observations of the inverse relationship between serum iron and TIBC in a variety of clinical situations, Lane (1966) suggested that the plasma iron level might control the regulation of transferrin synthesis.

In a series of animal experiments Morgan (1962, 1969) has suggested that the balance between oxygen supply and demand is the chief factor controlling transferrin synthesis. However he was unable (1970) to show that the TIBC rose in human subjects subjected to reduced atmospheric pressure for up to 4 days although there was a reticulocyte response. It is obvious that plasma transferrin concentration is a resultant of increased synthesis and catabolism and as these two processes may be controlled independently interpretation of static plasma concentrations may be misleading and it is essential to study both components simultaneously to gain a true picture. Furthermore there is evidence from distribution and turnover studies with radioactive labelled protein that a rise in plasma transferrin may sometimes be due at least in part to a shift from the extravascular to intravascular pool (Morgan, 1966). Factors of a more general nature may also be important in controlling synthesis. Protein deprivation reduces transferrin as well as albumin synthesis (Morgan and Peters, 1971a) and this has its clinical counterpart in kwashiorkor where low serum transferrin concentrations are a prominent feature (McFarlane et al, 1970). In inflammatory states the low plasma iron and iron-binding capacity are well known and studies in man (Jarnum and Lassen, 1961) and in the rat (O'Shea et al, 1971) show that while synthesis is well maintained fractional catabolism is increased reducing the plasma pool. Oestrogen therapy is accompanied by a rise in serum transferrin (Laurell et al, 1967; Jacobi et al, 1969; Song et al, 1970) and levels also rise in the last trimester of pregnancy but in this situation iron deficiency may also play a part (Mendenhall, 1970). Anabolic steroids do not affect plasma levels

(Barbosa et al, 1971). Extremely low synthetic rates may be found in the rare cases of congenital deficiency (Heilmayer et al, 1961; Goya et al, 1972) although the possibility of failure of release of the protein from the liver analogous to the situation in α_1-antitrypsin deficiency has not been explored. Excessive loss of the protein in the urine may rarely severely reduce plasma concentrations (Heilmeyer et al, 1966).

It has recently been reported that in normal subjects a small proportion of the serum iron is bound to other proteins (van der Heil, et al, 1972) and amino acids (Prasad and Oberleas, 1971) but the error in most circumstances in assuming that the TIBC is a measure of plasma transferrin is small (Stokceski et al, 1965; Dobrilla et al, 1967). However in disease states in which the plasma protein and amino acid pattern is greatly disturbed this may be a source of error. In addition the usual method of measuring TIBC which depends on removing excess unbound iron with magnesium carbonate will not precipitate out iron in the form of ferritin. The extremely high serum iron levels sometimes found in acute hepatic necrosis and in haemochromatosis (Brendstrup, 1953; Bothwell and Finch, 1962) are due in part to circulating ferritin (Reissmann and Dietrich, 1957; Jacobs et al, 1972) and the measurement of TIBC may overestimate the true serum transferrin concentration. This has been shown by Dobrilla et al, 1967; Fiaschi et al, 1969; Cartei et al, 1970, but the spectrophotometric method used for measuring TIBC may not have been entirely satisfactory.

The true levels of serum transferrin in haemochromatosis were low in the second series of patients in this study and this is in keeping with reports by Blanc and Vannotti (1966), Dobrilla et al (1967), Fiaschi et al (1969) and Cartei et al (1970). The latter two groups have also reported that levels rose to normal during venesection but it is difficult to know to what extent this may be due to the improvement in liver function

which generally occurs (Williams et al, 1969) rather than to removal of iron in particular. A rise in serum transferrin concentration during venesection was seen in three patients in the present series. Studies on the effects of regular plasmapheresis in normal volunteers showed that removal of about 1.2l plasma per week had no effect on the concentration of transferrin and other proteins (Shanbrom et al, 1972) but removal of 5l. per week did reduce plasma protein levels (Kliman et al, 1964).

The transferrin levels in the 14 relatives of the patients with haemochromatosis in the present series were significantly lower than the control population. There did not appear to be any relationship with the serum iron or iron stores. Blanc and Vannotti (1965) noted that 4 brothers of a patient with primary haemochromatosis had low plasma transferrin levels in addition to raised levels of serum iron and Dobrilla et al (1967) found 5 relatives of one of their patients had raised serum iron levels and low concentrations of plasma transferrin. However the normal range for TIBC is wide and the number of cases studies is small and although the mean TIBC was lower than in controls in 23 relatives studied by Williams et al (1962; 1965) and 52 relatives by Powell (1965) the differences were not significant. The mean TIBC in those relatives with significant iron overload assessed by a chelation technique was lower than in those with normal iron stores but the difference did not achieve statistical significance (Powell, 1965).

In agreement with other published studies we found normal or only slightly reduced transferrin levels in acute viral hepatitis (Muller and Muller von Voigt, 1967; Kindermark and Laurell, 1972), in primary biliary cirrhosis (Hallen and Laurell, 1972). Normal or slightly reduced levels were found in cirrhosis of unspecified type or in a mixture of types by Muller and Muller von Voigt (1967), Dobrilla et al (1967) Brezin et al (1971) and Weeke (1973). In patients with alcoholic cirrhosis in the present series

low transferrin levels were found and similar results are given by Fiaschi (1969) and Aron et al (1971). Normal levels were found by Hallen and Laurell (1972). The poor nutritional state of many alcoholic patients may contribute to the low levels and it is noteworthy that the serum albumin level was lower in this group than in the other types of cirrhosis studied. The fall in transferrin in obstructive jaundice is in keeping with the fall in TIBC reported by Lange and Oberhoffer (1958). The raised levels of transferrin in active chronic hepatitis were unexpected and have not been commented on in the literature. However in Hallen and Laurell's (1972) series of patients with acute hepatitis raised levels were found in over half the cases at 5-8 weeks when liver function was recovering. Such an increase, presumably due to increased synthesis, was also seen in 1 of the 4 cases of active chronic hepatitis followed serially during treatment and was also found in various experimental inflammatory states (Morgan, 1969). Cartei et al (1970) and Horne and MacSween (1971) have commented on the close correlation between albumin and transferrin both in normal subjects and in patients with liver disease. In the present studies it was not possible to confirm this observation but it is interesting that the two groups with the lowest mean serum albumin (patients with alcoholic cirrhosis and extrahepatic obstructive jaundice) were those which also had a low mean serum transferrin concentration.

The low levels of transferrin in the patients with haemochromatosis, alcoholic cirrhosis and extrahepatic obstructive jaundice may not be of any pathophysiological importance. Certainly there did not seem to be any marked tendency to infection. It is interesting however that iron overload is a feature of the two chronic states. No assessment of the body iron stores of the patients with alcoholic cirrhosis was made but a moderate degree of iron overload is common in this condition (Williams et al, 1967).

In a study of hepatic siderosis in patients with chronic liver disease (Williams et al, 1967) it was not possible to relate the degree of iron overload in the liver to increased absorption, degree of disturbance in liver function or serum iron. However the percentage saturation of the TIBC was significantly higher in those patients with siderosis reflecting a reduction in serum transferrin levels. The low level of transferrin must mean that for a given serum iron a higher proportion than normal of the transferrin molecules carry 2 atoms of iron and recent work by Fletcher (1971) has shown that iron in this state is preferentially delivered to the liver. This confirms earlier observations by Whoby and Jones (1963). Thus the low transferrin levels might be a factor in the development of iron overload in chronic liver disease.

β Lipoprotein

There is now good evidence from studies on the incorporation of radioactive labelled amino acids and lipids that the liver is the major site of β lipoprotein (LDL) synthesis, with lesser amounts arising from the intestine (Hatch et al, 1966; De Jong and Marsh, 1968; Marsh, 1971; Stein et al, 1972). The presence of LDL peptides in the VLDL fraction raises the question of whether the LDL are secreted as such by the liver or whether the peptides first appear as VLDL. This is at present unknown (Marsh, 1971). The plasma concentration of β lipoprotein is influenced by both environmental and genetic factors as saturated fats raise the serum cholesterol and LDL when fed by isocaloric substitution for carbohydrate and other fats, and elevated levels are commonly familial (Lewis, 1971). Hyperbetalipoproteinaemia is also found in hypothyroidism, idiopathic hypercalcaemia and the nephrotic syndrome. A slight drop in concentration occurs as part of the acute phase reaction.

In liver disease the changes in lipoprotein concentration found depend on the methods of investigation used. Early work with moving boundary or

paper electrophoresis showed an increased β globulin fraction in obstructive jaundice which was attributed to lipoprotein (Sterling and Ricketts, 1949; Kunkel and Ahrens, 1949; Kunkel and Slater, 1952). When agarose electrophoresis is followed by lipid staining there appears to be a reduction in α lipoprotein and pre-betalipoprotein (Papadopoulos and Charles, 1970; Wollenweber and Kahlke, 1970; Seidel et al, 1972). However HDL can be demonstrated immunologically in the serum and ultracentrifugal analysis has shown that the VLDL is also present but has β mobility on electrophoresis. This pattern of changes is found in all types of liver disease and Seidel and his co-workers (1972) have shown that it is due to an abnormality in the apolipoprotein A. This results in decreased lipid binding by the HDL and absence of the usually small quantity of the apolipoprotein A from the VLDL is accompanied by a change in mobility from pre-beta to beta. Ultracentrifugal analysis of LDL in obstructive jaundice has shown increased levels associated with raised serum cholesterol and phospholipid levels. Early work (Eder et al, 1955) based on the composition of Cohn fractions of plasma from patients with obstructive jaundice had suggested the presence of an abnormal lipoprotein and this protein has recently been more closely characterised and shown to account for much of the increase in the LDL fraction (Switzer, 1967; Seidel et al, 1969). This abnormal lipoprotein known as lipoprotein-X (LP-X) has a unique composition and is rich in phospholipid and unesterified cholesterol. The protein moiety consists of albumin and apolipoprotein C. (Seidel 1972).

It is not possible to be certain what ultracentrifugal classes of lipoprotein were being measured as β lipoprotein by the present method. As apolipoprotein B is the major protein component of both the VLDL and LDL and has one antigenic determinant (Alaupovic, 1972) the β lipoprotein arc may represent both ultracentrifugal types. However it is unlikely to include LP-X which contains mainly apolipoprotein C and does not react

with antisera to normal lipoprotein. Hence, although the LDL fraction increases in obstructive jaundice the rise is due largely to lipoprotein-X and it is therefore not surprising that β lipoprotein levels measured immunologically in the present study were normal in primary biliary cirrhosis and low in extrahepatic obstruction. The change in the electrophoretic mobility of the VLDL from prebeta to beta which occurs in liver disease might be expected to alter the configuration of the precipitation arc but no difference was detected between normal subjects and patients with liver disease.

Levels of β lipoprotein were normal in all groups of liver disease studied except for acute viral hepatitis where the concentration was significantly raised. Kindmark and Laurell (1972) using the same immuno-electrophoretic technique noted normal or raised levels in patients with acute viral hepatitis but no details were given. Other studies of lipoprotein measured by immunological methods in liver disease showed normal levels in alcoholic cirrhosis (Weeke, 1973) raised levels in a small series of patients with primary biliary cirrhosis (Weeke, 1973) and low or normal levels in unspecified or mixed types of cirrhosis (Muller and Muller von Voigt, 1967; Brezin et al, 1971; Weeke, 1973).

Third component of complement (C3)

Evidence that C3 is made in the liver comes from studies on the in vitro incorporation of amino acids (Kaghn and Prince, 1971) and immuno-fluorescence in liver slices produced by fluorescein tagged specific antibody (Johnson et al, 1971). In addition there is abundant evidence in experimental animals and also in man that cells of the lymphoid series have the capacity to synthesise this protein (Hochwald et al, 1961; Phillips and Thorbecke, 1966; Stecher and Thorbecke, 1967; Glade and Chessin 1968). However the change in C3 phenotype following liver transplantation is strong evidence in favour of the liver being the primary site of synthesis (Alper

et al, 1969).

Kohler and Muller-Eberhard (1967) measured total C3 (β_1 AC) levels by immunodiffusion at intervals over 60 days during conversion at 20°C and found the total amount rose by 21%. This was due to the loss of the B antigen on conversion to β_1A and the immunoprecipitin ring formed by the β_1A precipitating with anti A antibody is larger giving an apparent increase in the concentration of C3 in the serum (Laurell and Lundh, 1967; West et al, 1967). This artefact would influence the results of β_1AC measured by the present technique because the serum samples were stored for variable periods of time. The conversion could have been run to completion by incubating the serum at 37°C. for 3 days but this might have altered the concentrations of other plasma proteins. In the first transplant patient where the storage of serum was brief and comparable with the control sera, the values for the concentration of β_1AC globulin are probably valid. The 50-80% increase in the concentration of β_1AC globulin in the patients with cryptogenic cirrhosis, alcoholic cirrhosis and active chronic hepatitis may in part be due to the storage artefact but this increase is more than can be attributed to conversion to β_1A alone. Because of the small size of the precipitation arcs on the miniplates no attempt was made to measure β_1AC globulin.

There have been several studies recently of C3 levels in liver disease. In acute hepatitis there is often a fall initially and when the jaundice reaches its peak the levels are normal or above normal (Onion, et al, 1971; Kosmidis and Leader-Williams, 1972; Alpert et al, 1972). This suggests consumption of complement possibly due to antigen-antibody complexes but it occurred in both Australia antigen positive and negative cases and the pattern was the same whether or not there were symptoms such as arthritis suggesting immune complex disease. Recent evidence supporting consumption

of complement by immune complex formation comes from studies in this Unit on patients with Australia antigen positive chronic liver disease, who have been given an intravenous infusion of gamma globulin containing a high titre of antibodies to Australia antigen. Following the injection there was a fall in total haemolytic complement, C3 and C4 with evidence on immunoelectrophoresis of in vivo conversion of C3 (Drs. A. Eddleston and W. Reed, personal communication).

C3 levels are also low in massive hepatic necrosis and gradually rise with improvement in liver function. Here it is likely that impaired hepatic synthesis is responsible for the changes (Fox et al, 1971). In all types of chronic liver disease most reports comment on the wide scatter of values (Agostini et al, 1968; Grob et al, 1971). In patients with high levels Kaboth and Arnold (1969) found evidence of marked chronic inflammatory cell infiltrate and suggested the rise in C3 was part of the acute phase reaction. Other authors have not commentedoon this but have found no relation to auto-antibodies or γglobulin levels (Grob et al, 1971; Finlayson et al, 1972). Low values tend to occur in patients with severely disturbed liver function tests and both Kaboth and Arnold (1969) and Finlayson et al (1972) found a positive correlation between C3 levels and coagulation tests. Measurements of Cl_q, which is synthesised outside the liver, were normal in 11 cases of liver disease of which 8 had low levels of total haemolytic complement C3 or C4 (Finlayson et al, 1972). This is further evidence for diminished hepatic C3 synthesis as the cause of low C3 levels but activation of the alternate pathway (Peters et al, 1972) will not affect Cl_q levels and complement fixation.by immune complexes remains a likely explanation at least in some cases.

Thus it appears that there are at least 2 mechanisms for the finding of low serum complement in liver disease. One mechanism is the activation

of serum complement by immune complexes and the other is a low rate of synthesis in severe hepatocellular disease. The latter mechanism has been confirmed in a few cases of advanced cirrhosis by measuring the plasma disappearance rate of ^{125}I labelled C3 (Petz 1971). Increased C3 turnover can of course be compensated for by increased synthesis so that plasma concentrations are normal (Carpenter et al, 1969; Sliwinski and Zvaifler, 1972).

An alternative and easier method of detecting increased complement utilisation is to look for breakdown products as evidence of in vivo activation. These were first shown in vivo in certain types of glomerulonephritis by Soothill (1965) and further reports (Soothill, 1967; West et al, 1967; Alper and Rosen, 1967; Peters et al, 1972).have confirmed this. However the significance of these products in vivo is still not clear for some cases had normal C3 catabolic rates in vivo using radioactive labelled C3 (Peters et al, 1972) and serum from these cases contains a factor (termed nephritic factor) which breaks down C3 in normal human serum. The present studies failed to show evidence of significant conversion of C3 in vivo in active chronic hepatitis or primary biliary cirrhosis. However these results are open to criticism on the grounds that the commercially available antiserum used had only antibodies to the A and B antigens so that α_2D (C3d) and other products could not be visualised. However in the great majority of cases in which in vivo complement conversion has been found β_1A has been present. Furthermore the breakdown products may be found intermittently and it is advised that repeated samples be examined (Versey et al, 1972). Teisberg (Personal communication) has found C3 breakdown products in fresh plasma from some patients with both active chronic hepatitis and primary biliary cirrhosis but was unable to relate this to the degree of abnormality of liver function tests or the presence of auto-antibodies or Australia antigen. Chandra (1970) found altered C3 components in 21 out of 30 patients

with Indian childhood cirrhosis and in all cases C3 levels were severely depressed.

Most but not all studies in renal transplantation have shown hypercatabolism of C3 with a fall in plasma concentration at the time of rejection (Carpenter et al, 1967; Carpenter et al, 1969; Yokoyama et al, 1972) and both immunoglobulin and complement are found in relation to vascular endothelium of the rejected organ (McKenzie and Whittingham, 1968; McPhaul et al, 1970; Ngu et al, 1971). This has also been shown in liver allografts (Andres et al, 1972). The findings in the first hepatic transplant patient studied were in favour of C3 consumption at the time of the early rejection episode and high levels were found later when the degree of impairment of liver function was considerably greater. Torisu et al (1972) have recently confirmed a fall in C3 and C4 concentration with acute rejection in 5 liver transplant recipients and shown that there was no change in the concentration with biliary obstruction. Similar results were obtained using total haemolytic complement (CH_{50}) in 4 other patients in the King's College Hospital transplant series (Pagaltsos et al, 1972) and it does seem that serial measurements of C3, C4 or CH_{50} may be of value in the differential diagnosis of jaundice at least in the first month after liver transplantation.

Other proteins

There are few data in the literature on easily precipitable glycoprotein (PGP) but it appears to be an acute phase protein and shows a rise after surgery (Clarke et al, 1971) and in patients with rheumatoid arthritis (Clarke et al, 1970b) tuberculosis (Clarke, et al, 1970a) ulcerative colitis and Crohn's disease (Weeke and Jarnum, 1971). It was significantly raised in the present series in patients with hepatitis, extrahepatic obstruction and primary biliary cirrhosis, this latter finding being in agreement with Weeke (1973). This author also found raised levels in

alcoholic cirrhosis and a group of 26 patients with cirrhosis which included 7 cases of active chronic hepatitis. Although levels were also found to be raised in patients with cryptogenic cirrhosis, alcoholic cirrhosis and active chronic hepatitis, it was only in the latter group that this reached statistical significance.

Protein 16 also appears to be an acute phase reactant and rose after hernia repair (Clarke et al, 1971) and in a subject who developed influenza. Aronsen et al (1972) noted raised levels of antichymotrypsin after surgery and they suggested that this protein was identical with protein 16 as identified by Clarke and Freeman (1968). Recent work confirms the identity (Clarke, H.G.M.; personal communication). Kindmark and Laurell noted slightly elevated levels of antichymotrypsin in some patients with acute hepatitis which may correspond with our findings but there are no reports in the literature on concentrations in other forms of liver disease. By contrast protein 18 was at normal concentrations in three of the four groups of patients studied and the low mean value in the extra-hepatic obstruction group was due to apparent absence of the protein from 4 of the 12 sera. The concentration of protein 10 tends to fall with surgical trauma (Clarke et al, 1971) but in the present study it was elevated in patients with acute hepatitis and also those with active chronic hepatitis although in this group it did not reach statistical significance. The coefficient of variation of this protein in the normal population is large probably due in part to difficulties with measurement as it gives rise to a small faint arc on immunoelectrophoresis. However, the rise which was found only in the two forms of liver disease with the greatest inflammatory component was quite striking. Further studies of this protein in a range of conditions are needed.

Following transplantation of the liver an early rejection episode occurred in three of the four cases and this was accompanied by the changes of the acute phase reaction. This was almost certainly at least in part due to the preceding surgical trauma for following operation the plasma protein pattern does not return to normal for about two weeks (Werner and Cohnen, 1969; Clarke et al, 1971). In the 3 survivors liver function improved and the protein pattern returned towards normal. In case 1 the concentration of all proteins measured was normal on day 56 and 3 to 5 weeks post-operatively there were only minor abnormalities in the plasma protein pattern in cases 3 and 4. However further deterioration in the plasma protein pattern occurred in all 3 cases due to chronic rejection (Case 1), carcinomatosis with hepatic deposits (Case 3) and extrahepatic obstruction (Case 4). In each case the changes, which were those of the acute phase reaction, were qualitatively very similar although variable in degree and there were no unusual features except the extent of the rise in protein 16 which was 3 times above the normal level in cases 3 and 4. Measurements of albumin and fibrinogen synthesis rates made by the ^{14}C-carbonate method in two other patients at intervals up to 13 months after transplantation at a time when liver function was normal, gave normal or high values for both proteins indicating excellent synthetic capacity (Rake et al, 1970).

The changes in plasma protein fractions following liver transplantation in the dog were studied by Kukral et al (1966) who showed a rise particularly in the α_2 globulins with increased incorporation of ^{35}S-methionine suggesting increased synthesis. Ischaemic damage to the graft (Kashiwagi et al, 1968) was followed by a fall in the concentration of many proteins including caeruloplasmin, α_2-macroglobulin and C3. These workers were unable to detect any consistent changes accompanying rejection but the immunoelectro-

phoretic technique they were using was only semi-quantitative. There are no reports of the changes in plasma protein patterns following liver transplantation in man with the exception of studies on the complement system (see above).

A change in the haptoglobin type of the recipient to that of the donor following liver transplantation has been reported by Merril et al (1964) and Kashiwagi et al (1968). This change had occurred within 2 days and persisted for the 64 day period of observation. A change in group component type was also reported by the latter group. We have examined sera before and after transplantation in 8 patients and demonstrated a change in haptoglobin type in 1, in group component type in 5 and in α1-antitrypsin type in 1. (Dr. Peter Cook). This is confirmatory evidence that the liver is the major source of these plasma proteins.

In a detailed study of the changes in 21 serum proteins following renal tranplantation (Weeke, 1971b,c) the usual post-operative changes occurred. During late rejection episodes in 4 patients only slight and statistically insignificant changes occurred. No details are given of liver function tests which may be disturbed in renal graft recipients (Evans et al, 1968).

Value of plasma protein measurements in the differential diagnosis of liver disease

The wide range of functions performed by the liver has invited the introduction of a large number of different liver function tests over the years and it has long been appreciated that the use of more than one test improves diagnostic accuracy (Maclagan 1947; Wang, 1953; Hill and Zieve, 1957). Measurement of total protein, albumin and globulin (Sherlock, 1946) or the standard 5 electrophoretic fractions when considered alone (Owen and Robertson, 1956; Wall, 1958; Osserman and Takatsuki, 1963) proved of little

help in the differential diagnosis of liver disease although the results of discriminant analysis might suggest that this technique has been underrated. Heremans et al (1955) used the values for the α_1, α_2, β and γ globulin fractions obtained from paper electrophoresis expressed as a percentage of the total protein in a discriminant function to reclassify 24 cases of hepatitis and 16 cases of obstructive jaundice into their diagnostic groups. This was achieved with an error of only 4.5% but no attempt was made to test the system with new cases. Charbonnier et al (1955; cited by Heremans) in examinàng the discrimant value of protein electrophoresis in the differential diagnosis of 197 cases of jaundice used a combined value for albumin and α_1 globulin as they were unable to separate these fractions clearly and they discarded values for γ globulin. Their error in reclassifying the cases into obstructive and hepatocellular disease was 45%. This is similar to the results in the present study where a larger number of diagnostic groups was examined. There have been other attempts to use various forms of discriminant analysis in the differential diagnosis of the jaundice patient using clinical and laboratory data which included plasma protein measurements. Burbank (1969) used the total globulin and Begon and Dhumeaux (1971) used albumin α_2 and γ globulin in their Bayesian model but there is no indication of the discriminant value of these particular measurements. In a multivariate discriminant analysis study employing 9 liver function tests in 17 patients with cirrhosis and 19 controls the combination of BSP transport maximum and γ globulin were found to give maximum separation between the two groups (Ramsoe et al, 1970).

The development of simple methods for identification of individual plasma proteins has given rise to diagnostic tests for Wilson's disease and the liver disease associated with deficiency of α_1 antitrypsin but until recently there were no studies on the discriminant value of the numerous other plasma proteins in the differential diagnosis of liver

diseases. Werner et al (1972) using a stepwise discriminant analysis compared the accuracy of paper electrophoresis with the immunological measurement of 14 plasma proteins including IgG, IgA and IgM in allotting normal subjects and patients with one of 6 diseases including hepatitis and cirrhosis to their correct diagnostic category. There was little difference between the accuracy of the two methods. The five most discriminant specific assays (IgA, albumin, IgG, α_1-acid glycoprotein and IgM) taken together were no better than the 5 electrophoretic fractions but the combination of both methods did improve the diagnostic effectiveness. It was not stated how the two methods compared when considering only the patients with hepatitis and cirrhosis nor which individual proteins had the most discriminant value.

In the present study the error in reclassifying the normal subjects and the 4 miniplate diagnostic groups was hadf as large as the error in reclassifying the patients with alcoholic and cryptogenic cirrhosis, and active chronic hepatitis. This is perhaps not surprising as the latter three groups are more similar in terms of the pathological process and the pattern of disturbance of liver function than are the other four groups studied. No individual protein emerged as being of particular diagnostic importance and the rank ordsr of proteins selected to reduce the diagnostic uncertainty varied not only between the analyses of the group of four diseases and the group of three diseases but also between the two discriminant functions used. The use of 13 proteins rather than 8 in the discriminant function slightly further reduced the error of reclassification.

It is obvious that cellulose acetate electrophoresis alone is of no value in the differential diagnosis of the 7 liver diseases studied here. However, as one might expect, when the diagnostic choice is reduced to groups of 3 or 4 diseases the results are improved and agree fairly closely

with the results of reclassification using 8 protein values from immuno-electrophoresis. The addition of information from cellulose acetate electrophoresis and 13 proteins from immunoelectrophoresis gives the best diagnostic accuracy and this underlines again the well recognised need for a battery of liver function tests for accurate diagnosis. It is interesting to note that when the cellulose acetate and immunoelectrophoresis results were combined the first 4 variables in rank order were immunologically distinct proteins. It seems likely that the inclusion of values for the immunoglobulins would further improve the diagnostic accuracy.

The two methods of statistical analysis gave similar results when up to 8 variables were included but as the amount of information increased the error using the stepwise linear discriminant function was less. This may be because this, unlike the Bayesian function, takes account of the correlations between proteins.

WORK FOR THE FUTURE

It seems unlikely to be profitable to extend this research to further diagnostic categories of liver disease in view of the essentially similar pattern of disturbance found so far in the different disease groups. However, the present work has raised various problems of interest worthy of further study.

The analysis of the results indicates which plasma proteins are of value in assigning individual patients to their correction diagnostic group. The present method of measuring these proteins simultaneously in a single sample, while it has the advantage of being fairly cheap, is too time consuming and technically difficult for routine application. It is easier and more practical to use radial immunodiffusion. The value of these measurements compared with the other clinical and laboratory data in reducing the diagnostic uncertainty will be assessed in a prospective manner with the sequential Bayesian diagnostic model currently in use in this hospital.

The low serum transferrin levels in patients with primary haemochromatosis and their relatives will be investigated further and an attempt made to correlate the serum levels with iron absorption and the degree of iron overload assessed by serum ferritin levels. This may be a better guide than the differential ferrioxamine test. Turnover studies with radio-iodine labelled transferrin before and after venesection in patients with primary haemochromatosis may give useful information on whether body iron stores indeed play a significant part in controlling transferrin synthesis.

The finding of abnormal concentrations of several proteins in the epileptic patients is of great interest. It is intended to do further serial studies in individual patients over a longer period of time with measurement of urinary glucaric acid excretion as an index of hepatic microsomal enzyme induction. Increased plasma protein synthesis may be demonstrated by

performing radio-active iodine labelled protein turnovers before treatment and after a steady state has been reached. Here it will be of particular interest to study haemopexin in view of its postulated role as an intracellular haem-binding protein possibly controlling the activity of several intracellular enzyme systems. The pathophysiological implications of the disturbances in protein concentration have not been explored but for instance the high α_2 macroglobulin levels may upset blood coagulation homeostasis and increase the risk of venous thrombosis. There is certainly much further work to be done on the long-term clinical effects of hepatic enzyme induction.

PART 7

CONCLUSIONS

CONCLUSIONS

(1) From the review of the literature it is concluded that the liver is the major source of the plasma proteins and changes in the concentration of individual proteins occur in liver disease.

(2) Clarke and Freeman's method of crossed immunoelectrophoresis is an accurate and reproducible method of measuring up to 14 individual plasma proteins in a single 4ul. sample.

(3) The micro-modification described in Part 2 is quantitative and reproducible and has the advantage of being considerably cheaper because of the small amount of antiserum needed.

(4) Storage of serum samples at -20°C. for up to 12 months does not alter the concentration of the proteins apart from β-lipoprotein which showed a fall of 30-50%.

(5) The mean plasma protein concentrations in a control population of 70 subjects and the variation with age and sex found in the present work agree closely with the published literature. This supports the validity of the present method of measurement.

(6) Plasma protein concentrations in healthy individuals show little variation over an interval of several months.

(7) Changes in the concentration of many plasma proteins occur in patients with liver disease. The pattern of disturbance is similar in the different diagnostic groups, although it differs in degree, the most striking changes occurring in those patients with the greatest abnormality of liver function tests. In patients with severe impairment of hepatic parenchymal cell function the concentration of many proteins including haemopexin may be low initially but increase

as liver function improves. It is suggested that the basic pattern of change is due to the 'acute phase reaction' but this may be modified by the limitation to synthesis imposed by the underlying liver disease which evoked this non-specific response. A high concentration of haptoglobin, normally a feature of the acute phase reaction, was not found in any group of patients and this may be due to the occurrence of mild haemolysis. Raised levels of α_2-macroglobulin, which are not a feature of the acute phase reaction, strongly suggest underlying hepatic fibrosis or cirrhosis.

(8) Haemopexin levels are normal in patients with primary haemochromatosis contrary to a previous report. Transferrin levels are reduced both in patients with primary haemochromatosis and their first and second degree relatives. This may be related to iron overload and in 3 of the patients with primary haemochromatosis low levels rose with venesection therapy.

(9) A fall in β_1-AC (third component of complement) following orthotopic liver transplantation may indicate a rejection episode but the pattern of changes of the other proteins is of no help in the differential diagnosis of abnormal liver function in the post-operative period.

(10) Evidence from the literature suggests that the low plasma levels of $\alpha_1$antitrypsin found in subjects homozygous for the Pi^Z allele are due to a fault in the release of the protein from the liver. The present work indicates that the problem does not extend to other liver produced plasma proteins.

(11) There are changes in the concentration of several plasma proteins in otherwise healthy epileptic patients being treated with long-term anticonvulsant therapy. The pattern of disturbance is quite distinct from the acute phase reaction. It may be related to hepatic microsomal enzyme induction.

(12) No individual plasma proteins have been found to be of particular diagnostic value for the groups of patients with liver disease studied. The error in reclassification of the cases using two different discriminant functions was minimised by including the values of all the plasma proteins measured. This was considerably more accurate than the results of reclassification using only data from cellulose acetate electrophoresis. However, the best diagnostic accuracy is attained by combining both methods. The present work supports the use of a battery of specific plasma protein estimations in the differential diagnosis of liver disease.

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APPENDIX A

PREPARATION OF REAGENTS AND OTHER MATERIALS

1. Agarose (L'industrie Biologique Francaise S.A.) was added to boiling deionised water to make a 2% solution and sodium azide was added (5/1000 W/W). It was boiled for 1½hrs filtered through gauze and stored till use in 20ml. aliquots in sterile screw cap containers.

2. Barbitone buffer (0.06M) was prepared in 10 1. batches:-

Sodium barbitone (Analar) 61.7g.
Calcium lactate (Analar) 3.84g.
Sodium azide (Analar) 500mg.
Deionised water (5 1.)

Using a glass electrode and concentrated HCl the pH of the buffer was brought to 8.6 and it was then stored at +4°C. till use.

3. Antiserum to whole human serum was raised in goats (Dr. T. Freeman and H.M. Clarke) by injecting antiwhole human serum in Freund's adjuvent.

4. Acetylated albumin was prepared by adding an excess of acetic anhydride to pure human albumin in 6% sodium bicarbonate at 0°C. After 10 minutes an excess of lysine solution (pH 7.0) was added. After exhaustive dialysis against physiological saline the concentration of protein was adjusted to 6mg./ml. The solution was subdivided into 1ml. lots and freeze dried.

5. Amido Black stain. An 0.1% solution was made up in alcohol-acetic acid wash (Methyl alcohol, deionised water and glacial acetic acid in the proportions 5:5:1 v/v).

6. Acetate buffer for the caeruloplasmin stain

37ml. 0.2M acetic acid + 463ml. 0.2M sodium acetate + 500ml. distilled water makes up 1 1. of 0.1M acetate buffer pH 5.7.

7. Sudan black stain.

1g. Sudan black/litre 60% ethanol incubated for 24 hours at 37°C. and then filtered and stored in a dark bottle. 0.1ml. 30% sodium hydroxide is added to 160ml. dye before use and the plate is soaked for 24 hours and then washed in 50% ethanol.

Appendix B.

ate No.	1	2	3	Av	%				
0 - Acetyl.									
Prealb.									
- Albumin									
- Oroso									
- a_1Lipo									
- a PGp									
- a AT									
CC									
a_2HS									
a_2Macro									
Caer									
9									
Hapto									
Hpx									
Trans									
3 Lipo									
3 1A-C									
45									
41									
47									
99									
101									
X									

APPENDIX C

CLINICAL DETAILS OF PATIENTS WITH ACTIVE CHRONIC HEPATITIS STUDIED SERIALLY DURING TREATMENT

Case 1. This 20 year old Jamaican joiner presented in July 1971 with a 4 month history of increasing jaundice. Apart from an isolated episode of uveitis 4 years previously he had no past medical history and had had no drugs or known exposure to hepatitis. His alcohol intake was moderate. Physical examination showed deep jaundice and hepatosplenomegaly.

Investigations showed a normal blood picture and the prothrombin time was 8 seconds prolonged. Liver function tests were:- serum bilirubin, 19mg./100ml., alkaline phosphatase, 260 I.U./l. (normal up to 80 I.U./l.) and aspartate aminotransferase, 210 I.U./l. (normal up to 50 I.U./l.). The total plasma protein concentration was 8g./100ml.; albumin 3.9g./100ml.; gamma globulin 2.9g/100ml; and caeruloplasmin concentration 35mg./100ml. (normal 20-40mg./100ml). Australia antigen was demonstrated in the blood by immunoelectrophoresis. Serum autoantibodies to mitochrondria and smooth muscle and antinuclear factor were absent. Liver biopsy showed chronic aggressive hepatitis with marked piecemeal necrosis and established cirrhosis.

Treatment was started with prednisone, 15mg./day and his further treatment is shown in Fig. 19. Large doses of corticosteroids were required to control the level of plasma bilirubin.

Summary A 20 year old man with Australia antigen positive active chronic hepatitis and established cirrhosis responding poorly to corticosteroid therapy.

Case 2. This 50 year old English housewife was well till December 1971 when she had an episode of jaundice lasting about 2 months. In February 1972 the jaundice returned and she was referred for investigation and treatment. There was no other past medical history and no exposure to drugs, injections or cases of hepatitis. Her alcohol intake was modest. Physical examination showed jaundice, numerous spider naevi and hepatomegaly.

On investigation the blood picture was normal and the prothrombin time prolonged by 3 seconds. The liver function tests showed:- serum bilirubin, 8.5mg./100ml.; alkaline phosphatase, 269 I.U./l.; aspartate aminotransferase 935 I.U./l.; Total plasma protein was 7.9g./100ml.; albumin 2.7g./100ml. and gamma globulin 3.34g./100ml. Antinuclear factor (1/160) and antibodies to smooth muscle (1/160) were present in high titre but mitochondrial antibodies were absent. Australia antigen was not detected in the blood. Liver biopsy showed active chronic hepatitis with prominent rosette formation and numerous necrotic cells. The architecture was disturbed and nodular regeneration present.

Treatment with prednisone 30mg./day resulted in a rapid fall in serum bilirubin and improvement in her condition (Fig.20).

Summary A 50 year old woman with active chronic hepatitis and cirrhosis who responded well clinically and biochemically to corticosteroid therapy.

Case 3. This man became addicted to heroin in 1966 at the age of 19 and was exposed to the risk of syringe transmitted hepatitis on numerous occasions. He became jaundiced for the first time in December 1970 when a diagnosis of Australia antigen positive hepatitis was made. His liver function remained abnormal however, and a liver biopsy in May 1971 showed chronic active hepatitis. He was referred to King's College Hospital in January 1972.

On physical examination he had numerous spider naevi and hepatomegaly. Investigations showed normal blood picture and prothrombin time. Liver function tests were:- serum bilirubin 9.2mg./100ml.; alkaline phosphatase 198 I.U./l.; aspartate aminotransferase 385 I.U./l.; total serum proteins 7.0g./100ml.; albumin 3.5g./100ml. and gamma globulin 1.4g./100ml. Serum autoantibodies were negative but Australia antigen was repeatedly detected in the serum by immunoelectrophoresis. Liver biopsy showed florid chronic aggressive hepatitis with rosettes and septum formation.

Treatment with azathioprine produced slow improvement in liver function tests (Fig. 21).

Summary; A 25 year old drug addict with Australia antigen positive active chronic hepatitis who responded slowly and incompletely to azathioprine.

Case 4. This 40 year old woman felt tired and unwell in June 1971 and became jaundiced for the first time in September 1971. There was no history of drug ingestion, injections or contact with cases of hepatitis. She gave no family history of liver disease. Her father died of chronic chest disease and her mother of rheumatoid arthritis and renal failure. Her two children were well.

On admission to King's College Hospital in December 1971 she was in hepatic pre-coma with fetor hepaticus and a flapping tremor. Jaundice was marked but there were no skin stigmata of chronic liver disease. The liver was enlarged but the spleen was not palpable.

Investigations showed normal blood picture with prolongation of the prothrombin time by 10 seconds. The serum bilirubin was 9mg./100ml.; alkaline phosphatase 220 I.U./l.; aspartate aminotransferase 335 I.U./l.; Total serum protein 7.8g./100ml.; albumin 29g./100ml,; gamma globulin 3.8g./100ml.; antinuclear factor (1/320) and antibodies to smooth muscle

(1/320) were present in high titre but mitochondrial antibodies and Australia antigen were not detected.

Azathioprine was started together with protein restriction and neomycin for the encephalopathy and because of the development in hospital of ascites sodium restriction and diuretics were also added. She was also given 225g. salt poor albumin over 2 days at one point during her hospital stay (Fig.22). By the time of her discharge from hospital her serum bilirubin had fallen to 2mg./100ml. and aspartate aminotransferase to 133 I.U./l. Her general condition and liver function tests continued to improve. At the time of her last visit in November 1972 she was clinically well taking a 60g. protein diet with no added salt, neomycin 1g. b.d., prednisone, 10mg. per day, azathioprine 75mg. per day, and spironolactone 50mg. b.d. Liver function tests on this occasion had however deteriorated a little with the serum bilirubin 4.7mg./100ml. and serum aspartate aminotransferase greater than 250 I.U./l.

Summary A 40 year old woman with active chronic hepatitis with autoimmune features who responded slowly clinically and biochemically to corticosteroids but who showed recent slight deterioration in liver function.

APPENDIX D

CLINICAL DETAILS OF LIVER TRANSPLANT RECIPIENTS

Case 1 This 41 year old man presented with abdominal pain and swelling. At laparotomy he was found to have a vascular primary hepatoma involving both lobes of the liver. He was referred to King's College Hospital for transplantation. Orthotopic transplantation was performed on 23rd September 1968 the donor being a 13-year-old boy who had died from head injuries. The immediate post-operative course was uncomplicated and he was ambulant by the fourth post-operative day and was discharged home on the seventeenth day. On the third post-operative day liver function tests were normal apart from serum bilirubin 1.8mg./100ml, but thereafter they became increasingly disturbed, the serum bilirubin reaching a peak of 13.4mg./100ml. and aspartate aminotransferase 233 Units/100ml. on the 21st day. Liver biopsy showed features consistent with rejection and his immunosuppressive therapy with prednisone, azathioprine and antilymphocyte globulin was temporarily increased (for details see Case 4 in Calne, et al, 1968). There was a progressive improvement in liver function the bilirubin falling to 2.2mg./100ml and he returned to work in the 6th week.

From the 9th week onwards there was a progressive rise in serum bilirubin (Fig. 29) to levels of 30-40mg./100ml. and alkaline phosphatase to 170 K.A. Units/100ml. and the serum transaminase fluctuated around 200 Units/100ml. Despite massive immunosuppressive therapy his cholestatic jaundice progressed and he developed a chest infection and fungal meningitis (for details see Case 4, Williams et al, 1969).

At necropsy (four and a half months after transplantation) the biliary and vascular anastomoses were well healed and there was no extrahepatic cause for obstructive jaundice. Liver histology showed dense cholestasis

and periportal and centrilobular fibrosis with early septum formation but no true cirrhosis. Branches of the hepatic artery had thickened walls with lipid filled macrophages in the intima, appearances very similar to those seen in chronic rejection of renal grafts. On the basis of these findings and the demonstration of immunoglobulin in the vessel walls by an immunofluorescent technique (Andres et al, 1972) the late phase of progressive jaundice was thought to be due to chronic rejection.

Case 2. This 17-year-old boy was admitted to King's College Hospital on December 12th 1968 with a diagnosis of subacute hepatic necrosis proceeding to cirrhosis. In November 1968 he had had his first episode of coma. He responded partially to protein restriction and neomycin but further deterioration occurred on December 11th and at the time of admission he was in deep coma with marked hepatic fetor. He was unresponsive to noise or pain stimuli and his pupils were widely dilated and unresponsive to light.

Orthotopic hepatic transplantation was done later that day and 12 hours following this the patient regained consciousness and he was talking on the second day. The E.E.G. showed striking improvement (Parkes, Murray-Lyon and Williams, 1970). He was not given immunosuppressive therapy because of the possible danger of reactivating the presumed viral hepatitis infection. On the fifth post-operative day his temperature rose and he became confused and the following day he deteriorated further and died suddenly. Liver function which had improved initially deteriorated markedly in the last few days (Fig.30; see also Williams et al, 1969, Case 8). Necropsy showed centrilobular haemorrhage and cell necrosis. Portal tracts were oedematous and contained a dense infiltrate of inflammatory cells including large lymphoid cells with pyroninophilic cytoplasm. The appearances were thought to be consistent with acute rejection.

Case 3. This 31 year old woman presented in May 1970 with abdominal pain and diarrhoea and on investigation was found to have carcinoma of the colon with hepatic metastases. In June the primary tumour was removed and in August because there was no evidence of spread outside the liver, orthotopic liver transplantation was performed. The post-operative course was uneventful except for an episode of early rejection accompanied by a transient rise in bilirubin to 2.5mg./100ml. (Fig.31). Liver biopsy on the fifth day showed mononuclear cell infiltration and foci of liver cell necrosis typical of acute rejection. Temporary increase in immunosuppressive therapy (prednisone 200mg./day and Actinomycin C, 3 doses of 200ug. and azathioprine 150mg./day) resulted in gradual return of liver function tests to normal.

The patient was discharged home on the 39th post-operative day taking 50mg. prednisone and 150 mg. azathioprine daily. Shortly after this she started to complain of pain in the left shoulder and dorso-lumbar region and began to lose weight. She died cachectic on January 10th 1971 and necropsy confirmed carcinomatosis. Histological examination of the liver showed no evidence of rejection.

Case 4. This 60 year old man presented in April 1971 with an eighteen month history of weight loss and more recent abdominal distension. Investigation in King's College Hospital in November 1971 showed a poorly differentiated adenocarcinoma in the liver, probably a cholangiocarcinoma. There was no evidence of extrahepatic tumour.

He underwent laparotomy on 25th December 1971 and as the tumour was found to involve both lobes of the liver orthotopic transplantation was performed. The biliary anastomosis was bile duct to bile duct over a tube (stent) which passed down 2cm. into the recipient's common bile duct and

brought out to the surface through the dome of the liver.

His general condition was satisfactory in the immediate post-operative period but the bilirubin remained around 5mg./100ml. On the 6th day the bilirubin rose to 12mg./100ml. (Fig.32) and bile drainage stopped. Following injection of contrast medium through the stent (no radiological evidence of obstruction) the bilirubin gradually fell and reached 5mg./100ml. on day 13. It was felt that the episode of jaundice had been obstructive in nature and no change was made in the immunosuppressive therapy (prednisone 60mg. and azathioprine 75mg./day).

His general condition remained good and the stent was removed on the 18th day. Following this he experienced abdominal pain and there was a progressive rise in serum bilirubin (Fig.32). On the 30th day laparotomy was undertaken and a cholecystenterostomy was performed. There was a temporary fall in serum bilirubin but his condition again deteriorated and the clinical course was marked by increasing jaundice, rigors thought to be due to cholangitis and renal failure. He died 51 days after transplantation.

At necropsy the biliary anastomosis was found to be infected and partly obstructed by a small piece of tubing originating from the stent. There were widespread infiltrates in most organs with Candida albicans which was also grown from blood cultures.

THE UNIVERSITY *of* EDINBURGH

)ate of form's completion..... Name: .....

)ate of protein sameple: ..... Hospital No.: .....

., 2, 3, 4; No.....

o.5 ex

0	Male
1	Female

No.6 Source

0	Out-patient
1	In-patient less than 1/52
2	In-patient more than 1/52
3	Patient not seen in Unit.

o.7 ace

0	British
1	Irish
2	European
3	Negro-African
4	Negro-West Indian
5	Asian
6	Other
Y	Not known

No.8 Height in cms

0	Less than 130
1	" " 135
2	" " 140
3	" " 145
4	" " 150
5	" " 155
6	" " 160
7	" " 165
8	" " 170
9	" " 175
X	175 cms and over
Y	N.K.

).9 eight n Kg

0	Less than 30
1	" " 40
2	" " 50
3	" " 60
4	" " 70
5	" " 80
6	" " 90
7	" " 100
8	" " 110
9	110 kg and over
Y	N.K.

No.10 Age on admission in yr

0	Less than 20
1	20-24
2	25-29
3	30-34
4	35-39
5	40-44
6	45-49
7	50-54
8	55-59
9	60-64
X	65 yr and over
Y	N.K.

.11 e at set of rrent lness years

0	Less than 14 yr
1	15-19
2	20-24
3	25-29
4	30-34
5	35-39
6	40-44
7	45-49
8	50-54
9	55-59
X	60 yr and over
Y	N.K.

No.12 Duration of illness to date of completing form

0	Less than 12 months
1	Less than 24 months
2	Less than 36 months
3	Less than 48 months
4	Less than 60 months
5	Less than 72 months
6	Over 72 months (6 years)
Y	N.K.

.13 esent upa- onal itus

0	Full-time employment
1	Part-time employment
2	Not employed
3	Unemployed - economic
4	Unemployed - due to illness
Y	N.K.

.14 ial ss - istrar neral

0	Student
1	Class 1
2	Class 2
3	Class 3
4	Class 4
5	Class 5
Y	N.K.

No.15 Menstrual status

0	Not applicable
1	Post-menopausal
2	Menstrual
3	Pre-Ovulatory
4	Post-Ovulatory
5	Oral contraceptives
6	Pregnant, first 6/12
7	Pregnant, last 3/12

o.16 arital tatus

0 Child under 14 yr
1 Single
2 Married now
3 Married once, now widowed/divorced
4 Separated
Y N.K.

o.17 amily istory. st degree elations

0 None of these
1 1 " "
2 2 " "
3 3 " "
4 4 " "
5 5 " "
6 6 " "
7 7 " "
Y N.K.

Similar illness (specify)
Diabetes mellitus
Skin pigmentation
Autoimmune disease
Bowel disorder U.C.
Bowel disorder Crohn's
Bowel disorder Other

).18 mily story of undice 1st gree lations

0 None
1 Viral hepatitis - acute viral
2 Viral hepatitis - serum
3 Drug induced
4 Congenital
5 Cirrhosis
6 Hemolytic
7 Obstructive
8 Other
9 More than one of these
X Hereditary
Y N.K.

.19 erage cohol take, st 10yr.

0 None
1 Up to 10 units/week
2 " " 20 units/week
3 " " 30 " "
4 " " 40 " "
5 " " 50 " "
6 51 or more units/week
7 Unreliable; ? under 30 units/week
8 Unreliable; ? over 30 units/ week
Y N.K.

1 unit = (1 pint of beer or 1 double measure spirits)

.20 st lical story ., iron rapy

0 None
1 Peptic ulcer
2 Iron therapy
3 Ileo-colitis
4 Pancreatic disease
5 Blood disease including anemia
6 Renal disease
7 Cardiac disease
8 Sarcoidosis
9 Other
X None of these
Y N.K.

21 t ical tory - od nsfu- n (pts last 5

0 None
1 -5
2 -10
3 -15
4 -20
5 -25
6 -30
7 -35
8 -40
9 41 or more
Y N.K.

No.22 Past medical history - jaundice

0 None
1 Viral hepatitis - avh
2 Viral hepatitis - serum
3 Drug-induced
4 Congenital
5 Cirrhosis
6 Hemolytic
7 Obstructive
8 Other
9 More than one of these
Y N.K.

No.23 Number of children	0	None	No.24 Age of youngest child in years	0	No children
	1	1		1	1 year
	2	2		2	2 "
	3	3		3	3 "
	4	4		4	4 "
	5	5		5	5 "
	6	6		6	6 "
	7	7		7	7 "
	8	8 or more		8	8 " "
	Y	N.K.		9	9 or more
				Y	N.K.

No.25 Symptoms in last 3 months	0	None of these
	1	Lethargy
	2	Abdominal pain (specify cause as final clinical diagnosis)
	3	Loss of libido
	4	Lethargy and loss of libido
	5	Pain and loss of libido
	6	Lethargy and pain
	7	All three
	Y	N.K.

o.26 ymptoms n last months	0	None of these
	1	Gum bleeding
	2	G-I bleeding
	3	Weight loss
	4	Gum and G-I bleeding
	5	Gum bleeding and weight loss
	6	G-I bleeding and weight loss
	7	All three
	Y	N.K.

o.27 bdominal welling, ain, dema	0	None of these
	1	Abdominal pain
	2	Abdominal swelling
	3	Ankle edema
	4	Abdominal pain and swelling
	5	Abdominal pain and ankle edema
	6	Abdominal swelling and ankle edema
	7	All three
	Y	N.K.

.28 amina- ion liver igmata	0	None of these	
	1	1 " "	Liver palms
	2	2 " "	Leuchonychia
	3	3 " "	Dupuytren's
	4	4 " "	Parotid enlargement
	Y	N.K.	

.29 amina- on trition	0	Obese
	1	Normal
	2	Poor nutrition (no wasting)
	3	Poor nutrition (must include wasting)
	Y	N.K.

.30 amina- on gmenta- on	0	No unusual pigmentation
	1	Pigmentation slight
	2	Pigmentation obvious
	Y	N.K.

.31 amina- on Neuro- ychiatric ndrome	0	None of these	
	1	1 " "	Fetor hepaticus
	2	2 " "	Liver flap
	3	3 " "	Reduced conscious level
	4	4 " "	Ataxia
	5	5 " "	(False) localising CNS signs
	Y	N.K.	

Examination e) Spider nevi
1 1 - 5 spiders
2 6 or more spiders
Y N.K.

No.33 Examination f) 2° Sex Characters
0 None of these
1 1 " "
2 2 " "
3 3 " "
4 4 " "
Y N.K.

Testicular hypotrophy
Loss of 2° sexual hair
Smooth facial skin (includes reduced shaving)
Gynecomastia

No.34 Examination g) C.C.F.
0 None of these
1 1 " "
2 2 " "
3 3 " "
4 4 " "
Y N.K.

Raised J.V.P.
Ankle edema in absence of ascites
Pulmonary edema
Abnormal rhythm/sounds

No.35 Examination h) liver edge below costal margin R.C.L.
0 Impalpable
1 Less than 2 cm, smooth
2 " " 4 " "
3 " " 6 " "
4 " " 8 " "
5 9 or more cm, smooth
6 Less than 2 cm, nodular
7 " " 4 " "
8 " " 6 " "
9 " " 8 " "
X 9 or more cm, nodular
Y N.K.

No.36 Examination i) Ascites
0 No ascites
1 Ascites doubtful
2 Ascites present
Y N.K.

No.37 Examination j) Spleen tip (cm below xiphisternum)
0 Impalpable
1 Less than 2 cm
2 " " 4
3 " " 6
4 " " 8
5 " " 10
6 11 or more cm
Y N.K.

No.38 Examination k) Peripheral neuropathy
0 None
1 Probable, legs
2 Certain, legs
3 Probable, arms
4 Certain, arms
5 Probable arms, certain legs
6 Both probable
7 Both certain
Y N.K.

No.39 Examination l) Arthritis
0 None detected
1 Rheumatoid polyarthritis
2 Spinal disease/S-iliitis
3 Other monoarthritis
4 Other polyarthritis
5 More than one of these
6 Hemochromatosis arthropathy only
7 " " and rhematoid disease
8 " " and other joint disease
Y N.K.

No.40 X-ray or biopsy confirmation of arthritis
0 Not confirmed
1 Confirmed by biopsy
2 Confirmed by X-ray
3 Confirmed by biopsy and X-ray
Y N.K.

No.41 Examination m) Skin lesions
0 Skin normal
1 Purpura
2 Xanthomata
3 Known skin disease (specify)
4 Nodular vasculitis
5 Perianal lesion(s)
6 Other
7 More than one of these
Y N.K.

No.42 Steatorrhoea (over 7 gm fat/day)
0 Not present
1 Present
Y N.K.

o.43
ecent
herapy -
teroids

0 None given
1 Still continuing
2 Ceased in last 3 months
3 " " " 6 "
4 " " " 9 "
5 " " " 12 "
6 " " " 15 "
7 " " " 18 "
8 " " " 21 "
9 Ceased over 21 months ago
Y N.K.

No.44
Current
therapy -
Steroids
(in mg
equiv.
Prednisa-
lone)

0 None given
1 Less than 10 mg daily
2 " " 20 "
3 " " 30 "
4 " " 40 "
5 " " 50 "
6 " " 60 "
7 " " 70 "
8 71 mg daily or more
Y N.K.

o.45
urrent
herapy -
iuretics

0 None given
1 Thiazides only
2 Aldosterone antagonists only
3 Other (specify)
4 Thiazides and other
5 Aldosterone antagonists and thiazides
6 Aldosterone antagonists and other
7 All three
X N.K.

o.46
ecent
herapy -
mmuno-
ppres-
ves

0 None given
1 Still continuing
2 Ceased in last 3 months
3 " " 6 "
4 " " 9 "
5 " " 12 "
6 " " 15 "
7 " " 18 "
8 " " 21 "
9 Ceased over 21 months ago
Y N.K.

No.47
Recent
therapy -
Venesec-
tion

0 Never
1 In last 1 month
2 " " 2 "
3 " " 3 "
4 " " 4 "
5 " " 5 "
6 " " 6 "
7 " " 7 "
8 " " 8 "
9 " " 9 "
X Over 9 months ago
Y N.K.

.48
tal gm
iron
moved
pto com-
etion of
rm)

0 None
1 Less than 5 gm
2 6-10
3 11-15
4 16-20
5 21-25
6 26-30
7 31-35
8 36-40
9 41 gm and more
Y N.K.

No.49
Current
Hb. gm%

0 Over 15
1 14-13
2 12-11
3 10-9
4 8-7
5 6-5
6 4-3
7 2 gm% and below
Y N.K.

.50
rrent
B.C.
tal

0 Up to 2,000 inclusive
1 " 4,000 "
2 " 6,000 "
3 " 8,000 "
4 " 10,000 "
5 " 12,000 "
6 " 14,000 "
7 14,001 and over
Y N.K.

No.51
Current
E.S.R.
mm/hr

0 0 - 4
1 5 - 9
2 10 - 14
3 15 - 19
4 20 - 24
5 25- 29
6 30 - 34
7 35 - 39
8 40 - 44
9 45 - 49
X 50 and over
Y N.K.

.52
itelets
)3

0 Less than 50
1 51-100
2 101-150
3 151-200
4 201-250

No.53
RBC size

0 Normal
1 Macrocytic
2 Microcytic
Y N.K.

).54 eticulo- yte count s %	0	0,1,2
	1	3,4
	2	5,6
	3	7,8
	4	9,10
	5	11,12
	6	13,14
	7	15,16
	8	17 and over
	Y	N.K.

).56 urrent erum A.P. -A units)	0	0-5
	1	6-15
	2	16-25
	3	26-35
	4	36-45
	5	46-55
	6	56-65
	7	66 and over
	Y	N.K.

.58 rrent rum K+ q/l	0	3.5 - 5.0 mEq/l
	1	0.5 - 1.9
	2	2.0 - 3.4
	3	5.1 - 6.5
	4	6.6 - 8.0
	5	8.1 - 9.5
	6	9.6 and over
	Y	N.K.

.60 rrent rum bili- bin mg% (II)	0	0 - 10
	1	10.1 - 20
	2	20.1 - 30
	3	20.1 - 40
	4	40.1 - 50
	5	50.1 and over
	Y	N.K.

.62 - etention 30 mins.	0	Less than 10%
	1	11 - 20%
	2	21 - 30 %
	3	31 - 40%
	4	41 and over
	Y	N.K.

.63 rent .T.	0	Normal
	1	Lag curve only
	2	Low threshold only
	3	Borderline
	4	Diabetic
	Y	N.K.

65 rent c acid	0	0.0 - 0.9
	1	1.0 - 1.9
	2	2.0 - 2.9
	3	3.0 - 3.9
	4	4.0 - 4.9
	5	5.0 - 5.9
	6	6.0 - 6.9
	7	7.0 - 7.9
	8	8.0 - 8.9
	9	9.0 - 9.9
	X	10.0 mg% and over
	Y	N.K.

No.55 Current prothom- bin time (sec. pro- longed over control)	0	2 or less
	1	3 - 4
	2	5 - 6
	3	7 - 8
	4	9 - 10
	5	11 or more
	Y	N.K.

No.57 Current Serum Na+ mEq/l	0	132 - 146 mEq/l
	1	90 - 103
	2	104 - 117
	3	118 - 131
	4	147 - 160
	5	160 - 174
	Y	N.K.

No.59 Current serum bili- rubin mg% (I)	0	0 - 1
	1	1.1 - 2
	2	2.1 - 3
	3	3.1 - 4
	4	4.1 - 5
	5	5.1 - 6
	6	6.1 - 7
	7	7.1 - 8
	8	8.1 - 9
	9	9.1 - 10
	X	Over 10.1 (See No.60)
	Y	N.K.

No.61 SGOT i.u.	0	0 - 30
	1	31 - 100
	2	101 - 300
	3	301 - 500
	4	501 - 700
	5	701 - 900
	6	901 - 1100
	7	1101 and over
	Y	N.K.

No.64 L.E. cells	0	Not detected
	1	Present
	Y	Not known

o.66
iver
iopsy -
ode.

0	No. 67	0	No. 68	0	
1	Liver	1	Liver	1	
2	biopsy	2	biopsy	2	
3	code.	3	code.	3	
4	Tens	4	Units	4	
5		5		5	
6		6		6	
7		7		7	
8		8		8	
9		9		9	
X		X		X	
Y		Y		Y	

).69
erum Fe
%

0	0 - 50 mg%
1	51 - 100
2	101 - 150
3	151 - 200
4	201 - 250
5	251 - 300
6	301 - 350
7	351 - 400
8	401 - 450
9	451 and over
Y	N.K.

No.70
Evidence
for portal
hypertension

0	None of these
1	1 " "
2	2 " "
3	3 " "
4	4 " "
Y	N.K.

Varices on Ba. swallow
Hematemesis or melena
Venous collaterals - abdominal wall ± hum
Other

.71
lenic
nographs
rgical

0	Normal
1	Portal vein thrombosis
2	Collateral channels
3	Both
4	Shunt - Splenorenal
5	Shunt - Portocaval
6	Portal v thrombosis and surgical shunt
7	Collaterals and spleno-renal shunt
8	Collaterals and PortoCaval shunt
9	Other
Y	N.K.

.72
tra-
lenic
essures
Hg

0	0 - 7
1	8 - 14
2	15 - 21
3	22 - 28
4	29 and over
Y	N.K.

No.73
Iron
Absorption

0	0 - 10 %
1	11 - 20
2	21 - 30
3	31 - 40
4	41 - 50
5	51 - 60
6	61 - 70
7	71 and over
Y	N.K.

74
mcg/Kg

0	0 - 200
1	201 - 400
2	401 - 600
3	601 - 800
4	801 - 1000
5	1001 - 1200
6	1201 - 1400
7	1401 - 1600
8	1601 - 1800
9	1801 and over
Y	N.K.

No.75
Immunology

0	No antibodies
1	Smooth muscle (SM)
2	Mitochondrial (M)
3	ANF
4	SM and M
5	SM and ANF
6	M and ANF
7	SM and M and ANF
Y	N.K.

76
and
EX

0	Both negative
1	DAT +
2	Latex +
3	Both +
Y	N.K.

No.77 Final diagnosis. Code I	0		No.78 Final diagnosis. Code II	0
	1			1
	2			2
	3			3
	4			4
	5			5
	6			6
	7			7
	8			8
	9			9
	X			X
	Y			Y
.79 Outcome 1 year.	0	Complete recovery		
	1	Chronic course - alive		
	2	Dead in 6 months		
	3	Dead in 9 months		
	4	Dead in 12 months		
	5	Dead, cause unrelated		
	Y	N.K.		
.80 Card No.	1			

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Serum Proteins after a Liver Transplant

PROTEIN concentrations after liver transplantation are of interest because most plasma proteins, other than the immunoglobulins, are made wholly or partly in the liver. The concentrations of eighteen immunologically distinct proteins have been measured by a quantitative immunoelectrophoresis technique[1] in a single subject before and for 4.5 months after receiving an orthotopic liver graft.

The recipient was a 41 year old man with a primary hepatoma and the donor liver came from a 13 year old boy, who died from head injuries. After the operation, carried out by Professor R. Y. Calne, the patient made a good recovery and was discharged on the seventeenth day. During the second week he showed some evidence of rejection, but after a temporary increase in immunosuppressive therapy he improved and was able to return to work in the sixth week. Unfortunately, he later developed progressive jaundice which was attributed to chronic rejection and died at 4.5 months from fungal meningitis[2].

The changes in serum protein concentrations observed led to the definition of two principal groups of proteins. Group (A) included those proteins which stayed at a normal or increased concentration from the time of transplantation to death. These proteins included haptoglobin, α_1 antitrypsin, caeruloplasmin, group component, and α_1 acid glycoprotein (Fig. 1). The concentrations of all these proteins were increased pre-operatively, probably because of necrosis in the tumour, and the concentrations remained high after the transplant. These proteins are often called acute-phase proteins, and Werner and Cohnen[3] showed that their concentrations increased after surgery, but returned to pre-operative levels within 18 days of uncomplicated surgical trauma. The persistence of increased concentrations in our patient beyond this time was probably a reaction to the early rejection episode. At the fifty-sixth day, the concentrations of these and the other sixteen plasma proteins measured were normal, but during the subsequent prolonged phase of chronic rejection, the acute-phase protein concentrations were again maintained above normal.

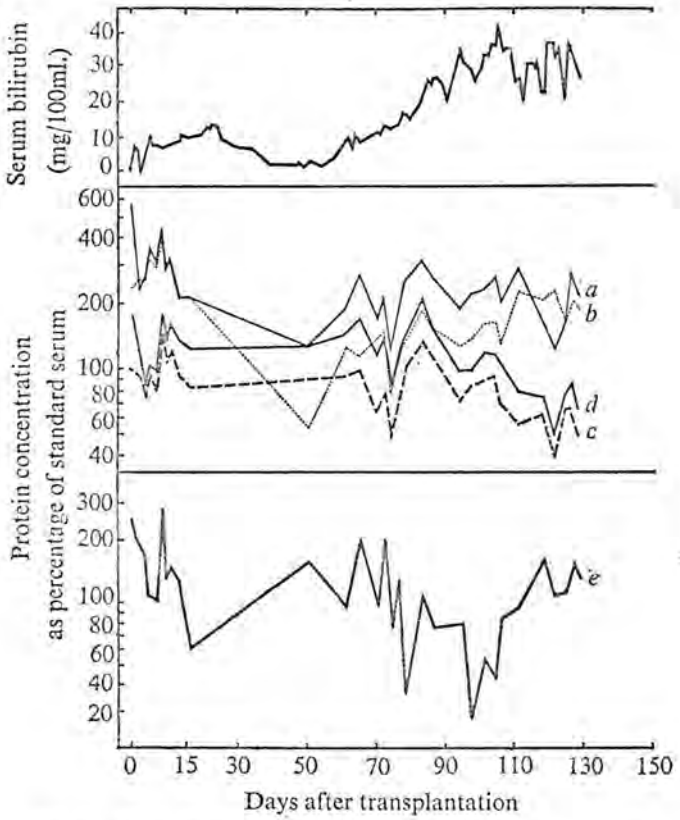

Fig. 1 Serial serum concentrations of haptoglobin (*a*), α_1 antitrypsin (*b*), albumin (*c*), transferrin (*d*) and β_1 A–C globulin (*e*) expressed as a percentage of a standard serum. Serum bilirubin concentrations are also shown.

Group (B) proteins included those that decreased gradually in concentration after the transplant, particularly after the eightieth day. They included albumin, transferrin and haemopexin. The α_1 lipoprotein concentrations also decreased throughout the study but at a greater rate than the other proteins mentioned. Because there was no reason for increased catabolism, the low concentrations of these proteins suggest either that there was no stimulus for synthesis or that the liver was unable to respond because of impaired parenchymal cell function. It is interesting therefore that at the same time the liver was able to maintain other proteins such as haptoglobin and α_1 antitrypsin at concentrations above normal. These changes may be a consistent feature of the acute-phase reaction[3,4].

The changes in two other proteins not included in our two groups are interesting. Thyroxine binding prealbumin behaved differently from the other proteins in that its concentration decreased initially, at the time of the marked increase in acute phase proteins, and again during the second increase in acute phase proteins after the eightieth day. Similar changes have been observed after simple surgery and virus infections[4]. The concentration of β_1 A–C globulin (the third component of complement C'3), which is probably synthesized solely in the liver[5], decreased significantly in the first 17 days at the time of the acute rejection episode (Fig. 1). At the beginning of the period of chronic rejection, the concentration was also greatly depressed but later, when infection may have been an important stimulus to synthesis, it increased to normal. Hypercatabolism of C'3, sometimes with a decline in serum concentration, has been shown during episodes of renal graft rejection[6] and this seems a more likely explanation than poor hepatic synthesis for the changes observed in our patient during rejection of the liver graft, as increased concentrations were found later in the clinical course when hepatic function was considerably impaired.

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NTITATIVE IMMUNOELECTROPHORESIS OF SERUM PROTEINS IN PTOGENIC CIRRHOSIS, ALCOHOLIC CIRRHOSIS AND ACTIVE ONIC HEPATITIS

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ived February 2, 1972)

IARY

Measurements of the concentrations of 10 serum proteins by quantitative im- electrophoresis, in 42 patients with inactive cryptogenic cirrhosis, alcoholic cir- s and active chronic hepatitis, showed that α_2-macroglobulin was increased in all diagnostic groups and caeruloplasmin in two—alcoholic cirrhosis and active ic hepatitis. In the latter condition transferrin and α_1 easily precipitable glyco- in were also elevated. Reduced concentrations of transferrin were found in al- c cirrhosis and of haptoglobin in active chronic hepatitis.

These changes showed little correlation with clinical data including liver func- ests. The greatest disturbance in plasma protein concentration was observed in chronic hepatitis and although the pattern was not typical, the mechanism may t be an "acute phase reaction" to inflammatory changes in the liver. The fall in globin is unexplained as is the mechanism of the rise in α_2-macroglobulin.

The finding of normal haemopexin levels in all three groups, is of interest in n to the marked increases previously reported in haemochromatosis, and further of this apparently specific abnormality is indicated.

UCTION

The majority of serum proteins are synthesized in the liver and certain patterns rbance have been described in different types of liver disease, although the stic value of conventional serum electrophoresis with separation into albumin e globulin peaks has proved rather limited[1]. Each of these globulin peaks in- may different proteins, and indeed by quantitative immunoelectrophoresis it ble to demonstrate the presence of at least 50 immunologically distinct serum s, although only a few can be identified with certainty. We have already re- the changes found in the sera of patients with haemochromatosis analysed by

TABLE I

MEAN PROTEIN LEVELS (± S.D.) EXPRESSED AS PER CENT REFERENCE SERUM IN THE VARIOUS CLINICAL GROUPS DESCRIBED IN THE TEXT

Protein	*Normal subjects*		*Cryptogenic cirrhosis*		*Alcoholic cirrhosis*		*Active chronic hepatitis*		*Haemochromatosis*	
α_1-Lipoprotein	127	(20)	108	(28)	89	(36)	101	(32)	139	(46)
α_1 Easily precipitable glycoprotein	115	(19)	139	(41)	140	(44)	151	(42)***	142	(27)
α_2 Group component	117	(15)	97	(37)	93	(20)	105	(35)	138	(41)*
α_2-Macroglobulin	122	(25)	170	(51)**	171	(61)**	175	(51)***	215	(68)***
Caeruloplasmin	137	(25)	166	(69)	177	(53)**	175	(45)***	173	(59)**
Protein 9	121	(17)	94	(51)	118	(23)	143	(57)***	Not measured.	
Haptoglobin	103	(44)	59	(84)	67	(63)	48	(52)***	113	(71)
Haemopexin	107	(13)	104	(36)	102	(29)	108	(38)	156	(49)*
Transferrin	110	(15)	114	(33)	91	(27)**	135	(39)**	119	(36)
β_1-Lipoprotein	177	(43)	157	(38)	198	(77)	175	(67)	146	(85)
Albumin (g/100 ml)	—	—	3.5	(0.56)	2.9	(0.42)	3.2	(0.66)	3.6	(0.64)

Statistical significance of difference between mean value in clinical group and that of normal subjects: $*p \leq 0.05$ $**p \leq 0.01$ $***p \leq 0.001$.

technique[2]. In this paper we describe an extension of that study to three other es of chronic liver disease.

ERIMENTAL

ients

The 42 patients comprised 12 with inactive cryptogenic cirrhosis, 12 with holic cirrhosis and 20 with active chronic hepatitis, of whom 13 had established hosis. In all but 2 cases liver biopsy was performed and separation between the ups was made according to current clinical, biochemical, histological and immuno-cal criteria[3].

trophoresis

The concentrations of 10 serum proteins, namely α_1-lipoprotein, α_1 easily pre-table glycoprotein, α_2 group component, α_2-macroglobulin, caeruloplasmin, pro- No. 9 (ref. 4), haptoglobin, haemopexin, transferrin and β_1-lipoprotein were sured using a modification[5] of Laurell's technique for quantitative immunoelec-horesis. Serum albumin was measured by standard paper electrophoresis.

istics

The protein levels for each patient were analysed statistically for correlations ngst proteins and between protein changes and the clinical data. This was repeated he 3 diagnostic groups so that relationships within the groups as well as in the le series could be determined. The mean protein levels in the 3 diagnostic groups also compared with those previously found in 100 normal subjects[6] and in those haemochromatosis already reported[2]. Standard statistical techniques were used ughout although adjustment of the distributions of all protein levels apart from oglobin and haemopexin was necessary as these appeared to be log-normal[6].

LTS

α_2-Macroglobulin concentration was significantly increased in each of the 3 clini-roups of patients (Table I). The patients with active chronic hepatitis also had icantly higher levels of α_1 easily precipitable glycoprotein, caeruloplasmin and ferrin. Caeruloplasmin was raised in the patients with alcoholic cirrhosis. In con- the only proteins to be significantly reduced were transferrin in alcoholic cir- and haptoglobin in active chronic hepatitis. The other five proteins namely opexin, α_2 group component, α_1- and β_1-lipoprotein and protein No. 9 showed no icant variation from normal.

In our previous study on 29 patients with haemochromatosis we found signifi-levations of α_2 group component, α_2-macroglobulin, caeruloplasmin and haem-n. When the mean values for these proteins were compared with the correspond-ures for the 3 groups of patients in the present study, the elevation of α_2 group onent and haemopexin in haemochromatosis was found to be significantly higher II). The mean level of haptoglobin in active chronic hepatitis was significantly than in haemochromatosis ($p \leq 0.001$). The scatter of values for the other pro-was wide and no other significant differences could be detected between the rent conditions for which data is now available.

TABLE II

COMPARISON BETWEEN THE MEAN PLASMA CONCENTRATIONS OF α_2 GROUP COMPONENT AND HAE PEXIN IN THE FOUR DIAGNOSTIC GROUPS

Values and symbols are as in Table I.

Groups compared	*α_2 Group component*	*Haemopexin*
Haemochromatosis with	138	156
cryptogenic cirrhosis	97***	104***
Haemochromatosis with	138	156
alcoholic cirrhosis	93***	102***
Haemochromatosis with	138	156
active chronic hepatitis	105***	108***

Significant positive correlations were found between α_1 easily precipita glycoprotein and α_1-lipoprotein, and between α_2 group component and haemope in active chronic hepatitis, alcoholic cirrhosis, cryptogenic cirrhosis and in haem chromatosis. In these 4 groups and in the normal subjects positive correlations w also noted between α_1 easily precipitable glycoprotein and both α_2-macroglobulin a haemopexin, and between transferrin and haemopexin. Significant negative corre tions ($p \leq 0.01$) were found between α_1 easily precipitable glycoprotein and albun in alcoholic cirrhosis and haemochromatosis.

An analysis of the protein levels with 15 indicants derived from the clinical d (these included clinical signs, evidence of portal hypertension and liver function te showed significant correlations only in the patients with active chronic hepatitis. this group the α_1 easily precipitable glycoprotein and transferrin were positively c related with serum alkaline phosphatase ($p \leq 0.001$).

DISCUSSION

Tissue damage or an inflammatory reaction, however caused, are known t accompanied by changes in the concentrations in the serum of certain proteins— so called "acute phase reaction". Levels of some proteins including haptoglobin, α_1-a trypsin, caeruloplasmin, α_1 easily precipitable glycoprotein and α_2 group compon rise, whereas the concentrations of others including transferrin, albumin and tryp phan α-rich prealbumin fall[7,8]. Various studies to-date have shown little change in macroglobulin and haemopexin following surgical trauma[7,8], although Laurell (sonal communication) found a slow rise in haemopexin following cholecystecto Although the most striking changes in protein concentrations were found in the tients with active chronic hepatitis and alcoholic cirrhosis, which of the 4 condit now investigated show the most marked inflammatory changes in the liver, the ov pattern of these changes was unlike the acute phase reaction. However, some of changes may be explicable on this basis.

Increased caeruloplasmin concentration has been reported previously in l disease[9] although in severe parenchymal impairment it may be reduced below no and this can cause diagnostic confusion with Wilson's disease[10]. The mechanis probably impairment of hepatic synthesis and this may also account for the sl reduction in transferrin and the more marked decrease of serum albumin concer tion in those with alcoholic cirrhosis. The poor nutritional state of these patients

an important factor as similar depression of albumin and transferrin is a striking ure of kwashiorkor[11].

The significant reduction of haptoglobin observed only in active chronic hepatitis its comment. In several patients the level was so low that there was no visible cipitation arc. A tendency towards low levels in cirrhosis has been attributed to chronic haemolytic state so often associated with liver disease, although Williams [12] could find no direct correlation with measurements of red cell survival.

Our previous finding of increased serum haemopexin concentration in haemomatosis needs to be considered in the context of the normal levels found in the r 3 types of chronic liver disease. Braun and Aly[13] found normal or low levels in osis but no details of the type of disease are given. Haemopexin transports iron e form of haem and belongs to the group of what have been termed "suicidal pro-", for after combining with haem the complex is catabolised, probably by the nchymal cells of the liver[14]. The high levels in haemochromatosis are unexplained may be due to the disordered iron metabolism. This protein is not usually coned to be an acute phase reactant but raised levels have been found in rheumatoid itis[15], tuberculosis[16] and inflammatory bowel disease[17].

The marked and consistent elevation of α_2-macroglobulin in all four types of disease is of considerable interest although its cause and significance are un-n, for the only certain functions of this protein are antiplasmin and antitrypsin ity[18]. Raised levels have also been noted by other workers in chronic liver se[18,19] although Müller and Müller-von Voigt[20] found normal levels in cirrhosis. ihepatic sites of synthesis may be important in maintaining these high levels[19].

A slight fall in α_1-lipoprotein levels with a normal concentration of β_1-lipoprogrees with previous findings in a group of patients with unclassified cirrhosis[20]. oup component was at normal concentration in our patients but reduced levels been reported in cirrhosis[19,20].

Although there do appear to be distinct differences in the pattern of abnormality , the relationship of changes in particular proteins to the underlying liver disease companying inflammatory response is far from clear. So far we have been un-o find much correlation between the changes in protein concentration and l or biochemical assessment of the severity of disease. To examine this further currently examining serial samples of sera from patients with active chronic tis both before and after treatment with corticosteroids or azathioprine.

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STUDIES BY QUANTITATIVE IMMUNOELECTROPHORESIS ON ON BINDING PROTEINS IN HAEMOCHROMATOSIS

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SUMMARY

1. The concentrations of a number of plasma proteins in patients with haemochromatosis were determined by quantitative immunoelectrophoresis, particular ttention being paid to the three iron transporting proteins.

2. Transferrin and haptoglobin were within normal limits; haemopexin was significantly greater than normal ($P<0{\cdot}001$).

3. Other changes observed were a significant ($P<0{\cdot}001$) increase of α_1 antitrypsin, f α_2 macroglobulin and β_{1A-C}; these changes are discussed.

st three serum proteins are involved in the ordinary physiological transport of iron. are, first, transferrin, which carries free iron. In normal circumstances transferrin is artly saturated, whereas in haemochromatosis it may be 90–100% saturated. Second, globin; this binds haemoglobin, and although it is known that the mass of haptoglobin lized per day is not enough to play a large role in the daily haemoglobin breakdown, it eivable that an upset in this mechanism could, over the years, account for an increase deposition of iron. And finally, haemopexin transports iron in the form of haem. Very known about the metabolism of this protein. It is thought (Müller Eberhard *et al.*, 1969) emopexin is a 'suicidal' protein like haptoglobin, in other words, after haem has comwith haemopexin the two are catabolized together. There is some evidence to support pothesis, in that a low haemopexin is sometimes found in haemolytic conditions; r, the concentration of haemopexin seldom drops as low as is found for haptoglobin. these reasons it seemed of importance to study the concentrations of these proteins in idiopathic haemochromatosis, a condition associated with a slow accumulation of iron tissues and eventual portal cirrhosis, and to compare the concentrations found with the normal population. At the same time the concentration of eight other proteins udied.

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MATERIALS AND METHODS

Twenty-nine patients with primary idiopathic haemochromatosis were studied. The diag of haemochromatosis had been confirmed in all the patients by liver biopsy which sh portal cirrhosis and Grade IV iron deposition according to the criteria of Scheuer, Will & Muir (1962). Many of them had been under observation for several years. All but one being or had been treated by weekly venesection for periods up to 3 years prior to the s

Protein estimation

Eleven proteins (α_1 lipoprotein (α_1Lp), α_1 easily precipitable glycoprotein (α_1PGp), α_1 trypsin(α_1AT), α_2 Group Component (α_2GC), α_2 macroglobulin (α_2M), caeruloplasmin(C haptoglobin (Hpt), haemopexin (Hpx), transferrin (Trf), β lipoprotein (βLp) and β_{1A-C} were measured according to the technique of quantitative immunoelectrophoresis as mod from Laurell's initial description (1965) by Clarke & Freeman (1968). Using this techniqu area contained under each individual protein curve is proportional to the concentration o protein in the serum. It is also inversely proportional to the concentration of antibody t particular protein; thus to make the technique quantitative it is necessary to use a single of antiserum throughout the study, and to use some form of standard. In this study the sta used was that originally described (Clarke & Freeman, 1968) obtained from a pool of human serum. The areas under the curves were measured using an electronic plani designed by J. Lewin of the National Institute for Medical Research, and manufactur Chemical Electronics Co. (C.W.S. Hall, Durham Road, Birtley, Co. Durham, England) values used throughout the study as normal were those obtained in the previous stud cribed by Clarke & Freeman (1968).

RESULTS

A photograph of a separation of normal serum is shown in Fig. 1 and that of a haemoch totic serum is shown in Fig. 2. Table 1 gives the values for the twenty-nine patients with h chromatosis and 100 normal subjects aged 16–65, giving means and SD. The sign differences obtained between the normal group and patients are shown.

DISCUSSION

The present study has shown highly significant differences in protein concentration b the patients with haemochromatosis and the normal subjects. It should be noted that ferrin, the only protein transporting free iron, is within the normal range. In the light concentrations obtained for other proteins, perhaps this is the most remarkable feature study. There are no significant correlations between the protein concentrations measur any of the clinical data, which included duration of venesection therapy and extent overload at the time of measurement. The exact relationship between tissue damage a overload is difficult to determine, and recently Smith *et al.* (1969) were unable to corre clinical findings at the time of presentation with the total body iron store measured differential ferroxamine technique.

A close relationship between haemopexin and the α_2 Group Component was found (*r*

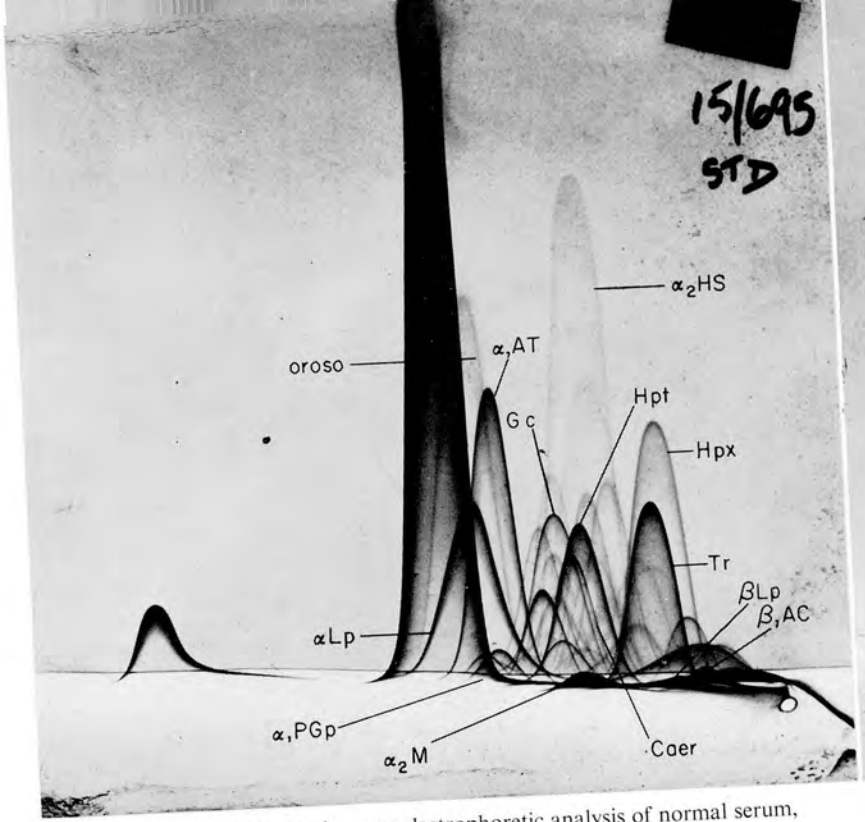

FIG. 1. A quantitative immunoelectrophoretic analysis of normal serum, showing α_1-lipoprotein (α_1Lp), α_1 easily precipitable glycoprotein (α_1PGp), α_1 antitrypsin (α_1AT), α_2 Group component (α_2GC), $\alpha_2$macroglobulin (α_2M), caeruloplasmin (Caer), haptoglobin (Hpt), haemopexin (Hpx).

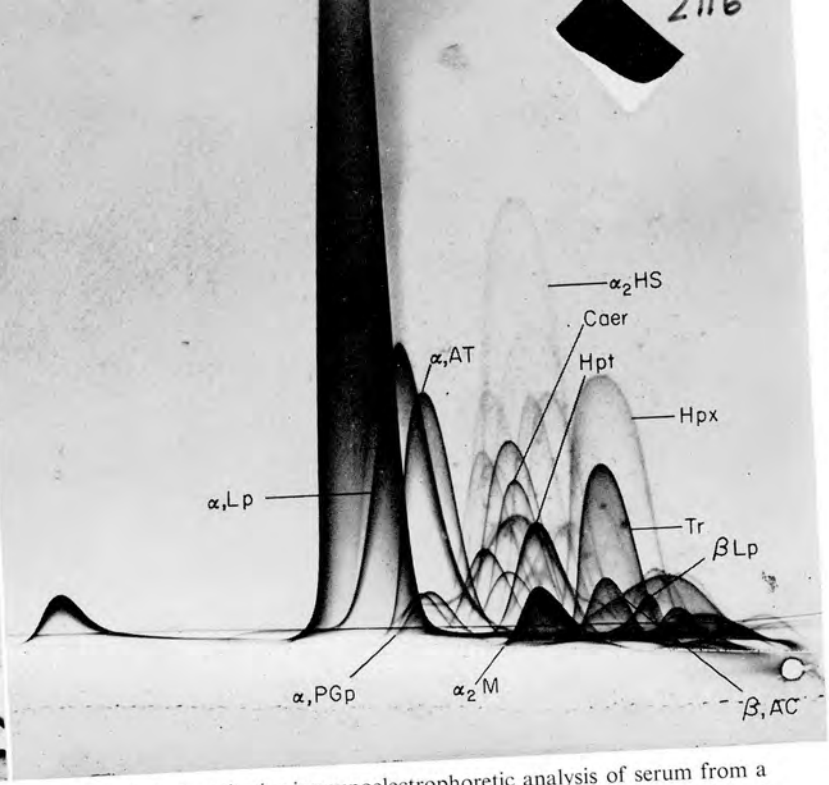

FIG. 2. A quantitative immunoelectrophoretic analysis of serum from a patient with haemochromatosis showing the same proteins as indicated in Table 1.

(*Facing p.* 614)

27, $P<0{\cdot}001$). The reason for this is not clear. The function of the α_2 Group component is nown, but similar correlations between these two proteins have been noted in other con-ns (Clarke, Freeman & Pryse-Phillips, 1970a). The interpretation of this finding must wait l more is known about the metabolism and function of Group component, and indeed of nopexin.

hilst it is appreciated that the measurement of concentration is not a valid way of assessing bolism of a protein, it would appear from this study that two of the three known iron porting molecules, transferrin and haptoglobin, are within normal limits, and that

TABLE 1. Protein concentrations (Mean Values and SD, expressed as per cent reference serum) in twenty-nine patients with haemochromatosis and 100 normal subjects

Haemochromatosis			Normals		Statistical significance of difference
Protein	Mean	(SD)	(SD)	Mean	
α_1Lp	139	(46)	127	(20)	—
α_1PGp	142	(27)	115	(19)	—
α_1AT	152	(39)	114	(18)	$<0{\cdot}001$
α_2GC	138	(41)	117	(15)	$<0{\cdot}05$
α_2M	215	(68)	122	(25)	$<0{\cdot}001$
Caer	173	(59)	137	(25)	$<0{\cdot}01$
Hpt	113	(71)	103	(44)	—
Hpx	156	(49)	107	(13)	$<0{\cdot}001$
Trf	119	(36)	110	(15)	—
β Lp	146	(85)	177	(43)	—
β_{1A-C}	155	(69)	107	(24)	$<0{\cdot}001$
n	29		100		—

pexin is significantly above normal. From measurements of concentration alone it t be said whether the increased concentration of haemopexin is due to an increase of sis or to a decrease in catabolism. Nor can it be said whether the increase is related to tiology of haemochromatosis or merely the resultant of a high body iron load at some

ificantly high values for haemopexin have been found in other conditions: in active ulosis ($P<0{\cdot}001$) but not in sarcoidosis (Clarke, *et al.*, 1970a); in schizophrenia ($P<$ but not in epilepsy (Clarke, Freeman & Pryse-Phillips, 1970b); and to a lesser extent in atoid arthritis ($P<0{\cdot}05$) (Clarke, Freeman & Pryse-Phillips, to be published). It could be that all four conditions (tuberculosis, schizophrenia, rheumatoid arthritis and haemo-atosis) show protein changes consistent with the 'acute phase reaction'. This ill-defined commonly used to describe that group of proteins which increase in concentration after damage. The concept is supported by an increase in α_1 antitrypsin in all four conditions ulosis, haemochromatosis and rheumatoid arthritis ($P<0{\cdot}001$), and schizophrenia 05); however, the changes in concentration in other proteins are not similar, for example plasmin is increased in tuberculosis ($P<0{\cdot}001$) and in haemochromatosis ($P<0{\cdot}01$) t in rheumatoid arthritis or schizophrenia, and α_1 easily precipitable glycoprotein is

normal in haemochromatosis and rheumatoid arthritis but raised in active tubercu ($P=0{\cdot}001$) and to a lesser extent in schizophrenia ($P<0{\cdot}05$). Finally haptoglobin, whi often quoted as an example of an 'acute phase protein', is normal in haemochromatosis increased in tuberculosis (mean 378, $n=16$, $P<0{\cdot}001$) and rheumatoid arthritis (mean= $n=18$, $P<0{\cdot}001$). It is however conceivable (though we think unlikely) that the normal centration found for this protein in haemochromatosis is the resultant of increased synt ('acute phase') and increased catabolism due to combination with haemoglobin. This p can only be decided by metabolic studies using trace labelled haptoglobin.

The large increase in α_2 macroglobulin seen in haemochromatosis is of some inte Although a controlled study of other diseases with primary liver damage is not yet comp it seems probable that similar high concentrations will be found. Again it is not clear wh this is due to increased synthesis or to decreased catabolism, though high protein conce tions are usually due to increase in synthesis. High concentrations of α_2 macroglobulin are found in the new born (Abrams & Freeman, 1969), in children (Abrams, 1970) and in y adults (Clarke & Freeman, 1968). The physiological reason for this is not known. Finall high significant increase ($P<0{\cdot}001$) in concentration of β_{1A-C} (the third component of plement: C'3) is unexplained. It would seem likely that this too is consequent to other p logical processes, and is not necessarily related to its complement activity.

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